Systematic inference of regulatory networks that drive cytokine-stimulus integration by T cells

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Abstract

Cell-fate decisions are governed by the integration of multiple stimuli. Th cell differentiation is a well-studied example of cell-fate decision: mature Th cells emerge from the thymus in a naive state and, upon encounter with their cognate antigen, differentiate into a specialised subtype depending on the polarising cytokines present in their environment. The most common and first described Th cell subtypes, Th1 and Th2 cells, differentiate in response to IFN-γ/STAT1 and IL-12/STAT4 (Th1) versus IL-4/STAT6 (Th2) signals, respectively, and start expressing the master transcription factors T-bet or GATA-3. T-bet in Th1 cells drives the expression of the effector cytokine IFN-γ that leads to the activation of macrophages, whereas GATA-3 in Th2 cells controls the expression of IL-4, IL-13 and IL-5, leading, among other things, to antibody class switching in B cells. Recent discoveries concerning the plasticity of Th cell subtypes as well as the existence of stable hybrid phenotypes, specifically T-bet$^+$GATA-3$^+$ hybrid Th1/2 phenotypes, have put Th cell fate decision under a different light. Those new findings stimulated the detailed study of the differentiation process under different assumptions than the hitherto valid paradigm of single master transcription factor expression deciding between alternative cell fates using complex cytokine signals as inputs in this work.

Here, we developed a data-based approach for inferring the molecular network underlying the differentiation of Th1, Th2 and hybrid Th1/2 lymphocytes. We performed systematic titrations of the polarising cytokines IFN-γ, IL-12 and IL-4 in all possible combinations during primary differentiation of *ex vivo* isolated naive Th cells and, using flow cytometry, quantified signal transduction in the form of STAT1, STAT4 and STAT6 phosphorylation as well as target-gene expression (T-bet, GATA-3, STAT1, STAT4 and STAT6 expression). Visual inspection of the thus generated dataset indicated the presence of a multitude of cytokine-specific regulatory mechanisms; however, the size and complexity of the dataset made a systematic analysis necessary to identify those mechanisms. To extract the network topology, we used linear regression analysis, retrieving known regulatory mechanisms and predicting numerous novel ones. The predicted network topology was verified experimentally and used to develop a mechanistic mathematical model of cytokine signal integration that reproduces independent data and confirms the significance of all predicted interactions.

This approach inferred a highly connected regulatory network. Previously undescribed functions of STAT signal transducers mediate network rewiring during differentiation: self-reinforcing feedback within the Th1 and Th2 regulatory pathways, self-limiting feedback within the Th1 pathway, and strong mutual inhibition

between the two pathways were predicted to be mediated by the phosphorylated STATs, and selected new interactions were confirmed by experiments using gene-deficient cells. Importantly, while mutual-inhibition motifs are often considered canonical digital switches, the inferred Th-cell network acts as a rheostat, generating a continuum of differentiated states along the Th1-Th2 axis. This work explains the observed Th1-Th2 cell fate continuum mechanistically and provides a quantitative framework for the data-based inference of cellular signal integration networks.

Zusammenfassung

Differenzierungsentscheidungen von Zellen werden durch die Integration mehrerer Stimuli bestimmt. Die Differenzierung von Helfer-T-Zellen (Th-Zellen) ist hierfür ein gut untersuchtes Beispiel: reife Th-Zellen treten in einem naiven Zustand aus dem Thymus aus und entwickeln sich beim Kontakt mit einem für sie spezifischen Antigen zu einem spezialisierten Subtyp, der von den in ihrer Umgebung vorhandenen Zytokinen abhängt. Die häufigsten und zuerst beschriebenen Th-Zell-Subtypen, Th1- und Th2-Zellen, differenzieren sich in Antwort auf IFN-γ / STAT1- und IL-12 / STAT4-Signale (Th1) bzw. IL-4 / STAT6-Signale (Th2) und exprimieren dann die Mastertranskriptionsfaktoren T-bet bzw. GATA-3. T-bet in Th1-Zellen treibt die Expression des Effektorzytokins IFN-γ, das zur Aktivierung von Makrophagen führt, während GATA-3 in Th2-Zellen die Expression der Zytokine IL-4, IL-13 und IL-5 steuert, die u. a. zum antikörper-Klassenwechsel in B-Zellen führen. Neuere Entdeckungen bezüglich der Plastizität von Th-Zell-Subtypen sowie die Existenz von stabilen Hybridphänotypen, insbesondere T-bet$^+$GATA-3$^+$ Hybrid-Th1/2-Phänotypen, haben die Schicksalscharakterisierung von Th-Zellen in ein anderes Licht gesetzt. Diese neuen Ergebnisse motivierten die detaillierte Untersuchung vom Differenzierungsprozessen von Th-Zellen in Anwesenheit komplexer Zytokinsignale in dieser Arbeit.

Dazu haben wir einen Ansatz für die datenbasierte Inferenz der molekularen Netzwerke entwickelt, die der Differenzierung von Th1-, Th2- und hybriden Th1/2-Lymphozyten zugrunde liegen. Wir haben systematisch die polarisierenden Zytokine IFN-γ, IL-12 und IL-4 in allen möglichen Kombinationen während der primären Differenzierung *ex vivo* isolierter Th-Zellen titriert und mittels Durchflusszytometrie Signaltransduktion (STAT1-, STAT4- und STAT6-Phosphorylierung) und Zielgenexpression (T-bet, GATA-3, STAT1, STAT4 und STAT6) quantifiziert. Die visuelle Auswertung des erzeugten Datensatzes legte die Existenz einer Vielzahl von Zytokin-spezifischen Regulationsmechanismen nahe. Der Umfang und die Komplexität der Daten machten jedoch eine systematische Analyse notwendig, um diese Mechanismen genau zu identifizieren. Lineare Regressionsanalyse wurde verwendet, um die Netzwerktopologie zu extrahieren. Dabei haben wir bekannte Regulationsmechanismen wiedergefunden sowie zahlreiche neue Interaktionen vorausgesagt. Die prognostizierte Netzwerktopologie wurde anschließend experimentell verifiziert und verwendet, um ein mechanistisches, mathematisches Modell der Zytokinsignalintegration zu entwickeln, das unabhängige Daten reproduziert und die Relevanz aller vorhergesagten Interaktionen bestätigt.

Diese Methode hat ein hochgradig venetztes regulatorisches Netzwerk inferiert. Bisher nicht beschriebene Funktionen von STAT-Signalwandlern vermitteln

die Neuverkabelung (rewiring) des Netzwerkes während der Differenzierung: Selbstverstärkende Rückkopplung innerhalb der Th1- und Th2-Regulationswege, selbstlimitierende Rückkopplung innerhalb des Th1-Signalweges und starke gegenseitige Inhibierung zwischen den beiden Signalwegen, die durch die phosphorylierten STATs getrieben werden, wurden vorhergesagt, und ausgewählte neue Interaktionen wurden in gezielten genetischen Experimenten bestätigt. Während gegenseitige Inhibitionsmotive oft als kanonische digitale Schalter interpretiert werden, funktioniert das Th-Zell-Netwerk als ein Rheostat, der Variationen der Zytokinsignale in graduelle Expressionsänderungen der Mastertranskriptionsfaktoren GATA-3 und T-bet übersetzt. Unsere Arbeit erklärt mechanistisch das beobachtete Kontinuum von Th-Zelldifferenzierungszuständen entlang der Th1-Th2-Achse und beschreibt eine quantitative Methode für die datenbasierte Inferenz zellulärer Netzwerke der Signalintegration.

Contents

Nomenclature

R^2 Coefficient of determination

AIC   Akaike Information Criterion

APC   Antigen-presenting cell

BCR   B cell receptor

CD   Cluster of differentiation

FoxP3 Forkhead box P3

GATA-3 GATA-binding factor 3

IFN   Interferon

Ig   Immunoglobulin

IL   Interleukin

JAK   Janus kinase

MAPK Mitogen-activated protein kinase

MHC   Major Histocompatibility Complex

MTF   Master transcription factor

NFAT Nuclear factor of activated T-cells

NK   Natural killer

ODE   Ordinary differential equation

pSTAT Phosphorylated signal transducer and activator of transcription

RORγt RAR-related orphan receptor γt

STAT   Signal transducer and activator of transcription

T-bet T-box expressed in T cells

TCR   T cell receptor

Tfh   T follicular helper

TF   Transcription factor

Th   T helper

Treg   T regulatory

Chapter 1

Introduction

1.1 The immune system and T helper cells

Organisms are under constant threat of infection by a multitude of pathogens[1]. All forms of life possess a defence system against pathogens, called immune system[2], that uses various mechanisms based on the same principle: the recognition and tolerance of self versus the recognition and attack of non-self. Pathogenic cells, like bacteria, yeast or helminth cells, can be identified by immune cells through detection of generic patterns present directly on pathogens, like bacterial lipopolysaccharides (LPS) or nucleic acids[3,4]. Infected or cancer host cells can be identified by the presence of foreign or modified antigen on the constitutively expressed major histocompatibility complex (MHC) molecules on their surface; healthy cells present self-antigen on their MHC molecules that induce tolerance by the immune system[5]. Furthermore, some healthy specialised immune cells, called antigen-presenting cells (APCs) can display foreign antigen on their surface to signal the presence of a pathogen to other members of the immune system[6].

Vertebrates possess a highly diversified immune system that is divided into two major branches[7]. The first barrier against pathogens, called the innate immune system, is strongly conserved amongst species and is a non-specific system that fights pathogens in a generic way, although different cell types act against different types of threat to the organism: type 1 immunity is active against most infections caused by small eukaryotic cells and is characterised by a high phagocytic activity, while type 2 immunity is active mainly against helminth and characterised by high antibody titers[8]. As its name suggests, innate immunity is already present and functional at birth; it has no memory, which means that its response will be the same if a pathogen is seen for the first time or has already infected the organism before. This system is able to control most of the infections directly at the interface between the organism and its environment through different simple mechanisms.

The second barrier, adaptive immunity, is present only in vertebrates[9] and is active if the innate immune response fails to contain an infection. The adaptive immune system fights pathogens in a specific manner: upon encounter with pathogenic antigen, a massive expansion of adaptive immune cells targeting the invading pathogen takes place (thus adapting the immune response to the detected threat). Fighting infection is a time-sensitive process which can be described as a contest between the pathogen multiplying in the host and the host destroying the pathogen; thus, a fast immune response is crucial for the survival of the host. Building an adaptive immune response takes times; However, so-called memory cells remain after the elimination of an infection by the adaptive immune system. These memory cells expand the pool of cells reacting to a specific antigen, thus increasing the efficiency of the adaptive immune response drastically[10].

1.1.1 The first line of defence: overview of the innate immune system

The innate immune system is the dominant immune response in plants and fungi but is also crucial for higher organisms. In order to fight a broad range of infections, from viruses to helminths, several mechanisms play a part in the mammalian innate defence: some are non-cellular, like the complement system, some, like anatomical barriers, are made by non-immune cells, and some require specialised cells. As a first line of defence, the innate immune system is responsible for preventing the entry of pathogens into the organism; failing that, for the clearing of the invading pathogens, and finally, if necessary, for the activation of the adaptive immune response. The principal players and functions of the innate immune system are:

Epithelial cells from the skin and mucosa secrete mucus and toxins that prevent the entry of pathogens into the body[11].

Inflammation aims at establishing a physical barrier around injured cells; those cells release chemical factors, like histamine, that promote vasodilatation and attract phagocytes to clear the pathogens[12].

The complement system is a protein cascade that leads to the opsonising or cytolysis of pathogens and triggers inflammation[13,14]; it complements the action of antibodies.

Professional immune cells all develop from the same hematopoietic progenitor cell, but differentiate into different cell types that have different roles[2]:

- Mast cells release inflammatory factors and chemokines to attract other immune cells[15].

- Phagocytes (macrophages, neutrophils and dendritic cells) engulf pathogens and unhealthy host cells and present pathogenic antigens on their MHC molecules so that other immune cells, specifically T cells, can be activated[16].

- Basophils and eosinophils secrete toxins against parasites and bacteria[17].

- Natural killer (NK) cells attack unhealthy host cells (recognising the 'missing self' condition, for example in cancer or virus-infected cells)[18].

1.1.2 A specific response to pathogens: the adaptive immune system

Sometimes pathogens expand faster than the innate immune response can control. When this is the case, a strong and specific immune response against the pathogen in question is needed; this is provided by the adaptive immune system. Adaptive immunity has a cellular and a humoral component. The effector immune cells, called lymphocytes, are divided into two main cell types: B and T cells. T cells mediate the cellular response, while B cells secrete immunoglobulins (Igs, also called antibodies) and take part in the humoral response. Both cell types originate from a common progenitor in the bone marrow that differentiates and matures in the thymus (for T cells) or in the bone marrow and spleen (for B cells)[19].

Both B and T cells recognise antigens through special proteins made of several chains that vary between clonal populations. T cells bind antigens via a T cell receptor (TCR) and B cells via a membrane-bound immunoglobulin (Ig) that forms the B cell receptor (BCR). Those receptors must have a very high affinity for one antigen to achieve the specificity of the adaptive immune response. This specificity is achieved by recombinations in the TCR and Ig gene loci; the T and B cells are mature once those recombination processes are finished[20,21]. In B cells, the immunoglobulin gene undergoes further mutations after an antigen encounter, called somatic hypermutations, which allow B cells to increase the affinity of their Igs for their cognate antigen at each encounter[22]. Once the maturation process is terminated, both T and B cells are considered naive as long as they have not encountered their cognate antigen, and have to be activated in order to become fully functional. This happens in a similar fashion for both cell types.

B cell maturation and function

B cells get activated when their BCR binds a free antigen and the cell receives a co-stimulus at the same time; the antigen is then engulfed, digested and presented as a peptide on a MHC class-II molecule at the cell surface. The peptide can then be recognised by T helper cells with the same antigenic specificity, which will secrete

cytokines allowing the B cell to mature into either an antibody-secreting plasma cell and to multiply or, for a small subset, into a memory cell[23]. Memory cells and long-lived plasma cells can survive for a long time[24], and react fast to a secondary infection thanks to their specific antibody epitope: no maturation process is needed and the antigen encounter is followed by a quick clonal expansion. Plasma cells derived from naive progenitors or memory cells secrete immunoglobulins with the same specificity as their BCR; those Igs act against pathogen in various fashions, the principal ones being:

Agglutination or precipitation is the process in which immunoglobulins bind several pathogenic antigens and join pathogenic cells (agglutination) or antigens (precipitation), thus facilitating their elimination by phagocytes;

Complement activation is the process that triggers the complement protein cascade;

Neutralisation is the process in which the binding of pathogenic antigens leads to a reduction in pathogen activity.

T cell maturation and function

T cells are key mediators of the cellular adaptive immune response. Progenitor T cells formed in the bone marrow migrate to the thymus where they expand and undergo maturation into T helper cells that express the cluster of differentiation (CD)4 marker or cytotoxic T cells that express the CD8 marker. The fate of T cells is determined, among other things, by the class of the MHC molecule they bind to during selection: cells interacting with MHC class-I molecules become CD8 T cells, while cells interacting with MHC class-II molecules become CD4 T cells[25]. The main role of cytotoxic T cells is to secrete cytotoxins leading to apoptosis of injured or infected cells. Perforin, one of the secreted cytotoxins, forms pores in the membrane of the target cell, allowing another kind of cytotoxin, granzymes, to enter the cell and activate the caspase cascade that causes apoptosis of the target cell[26]. The main role of T helper cells is to secrete cytokines activating other parts of the immune response against the pathogen carrying their cognate antigen[27,28].

Mature helper and cytotoxic T cells exiting the thymus are naive and have to be activated by antigens to become functional. Unlike B cells, T cells do not recognise free antigen; it has to be digested to peptides and presented on MHC molecules by antigen presenting cells (e.g., dendritic cells, macrophages, B cells, or non-professional antigen-presenting cells stimulated by interferon (IFN)-γ)[28,29]. Presented pathogenic peptides are bound by the TCR, which signals through the NFAT (Nuclear factor of activated T-cells) and MAPK (Mitogen-activated protein kinase) pathways, leading to the transcription of genes like *Tbx21* and

Gata3 encoding the so-called master transcription factors (MTFs) T-box expressed in T cells (T-bet) and GATA-3, as well as cytokine genes. The cytokine milieu during activation determines which genes are upregulated during this activation process, and thus the lineage into which the cells differentiate.

Immunological memory

The adaptive immune system has two hallmarks: the first one is the specificity of each cell for a defined antigen, and the second is that the system recalls pathogens it has already encountered. This is achieved by the presence of long-lived T and B memory cells that arise during the primary infection and survive in the absence of antigen[30,31,24]. These memory cells are already mature, having acquired a cytokine profile during differentiation for T cells and undergone class switching for B cells, making their response faster upon antigen re-encounter. Furthermore, the presence of memory cells increases the number of cells with a given antigenic specificity[10], thus making the expansion process much more efficient.

It is unclear why a primarily activated T cell will survive and enter the memory pool while most of its counterparts undergo apoptosis after the infection is cleared. However, interleukin (IL)-7 signalling play a crucial role by inducing the expression of B-cell lymphoma (Bcl)-2, an anti-apoptotic factor, in both CD4 and CD8 T cells[32,33], while the latter also need IL-15 and TCR signalling for the homeostatic proliferation of memory cells[34,35]. CD8 memory cells can be found in constant numbers in the spleen, while their CD4 counterparts reside in the bone marrow in the vicinity of IL-7-producing stroma cells[36].

1.1.3 Co-ordinating the global immune response: T helper cells

T helper cells are the main constituents of the CD4 population; their role is to activate other immune cells by secreting cytokines targeting the appropriate immune cell population to fight the pathogen. Hence, the mediators of their effector functions are those cytokines that signal to other cells (e.g., B cells, macrophages or neutrophils). Although innate immune cells act in defence against pathogens in the absence of T cell help, their response can be enhanced by the latter in case of an infection progressing despite their autonomous activity. In order to elicit a response adapted to the current threat to the organism, different Th cell types expressing different cytokines that target different effector populations arise in response to different pathogens[37,28].

CD4 T cells exiting the thymus are naive (with the exception of natural regulatory T (nTreg) cells[38]) and differentiate into one of several subtypes upon antigen encounter, depending on the cytokine milieu[39]. Differentiation into a specific subset is driven by polarising cytokines that signal mostly via Signal transducers

and activators of transcription (STAT) proteins[40], leading to the up-regulation of master transcription factors that drive effector cytokine expression. MTFs are defined by the fact that their expression is both necessary and sufficient to specify a Th cell lineage. MTF expression is sustained throughout the T helper cell's life and maintains the cell's phenotype in the memory phase. Commonly accepted subtypes are T helper type (Th)1, Th2, Th17, T follicular helper (Tfh) and induced regulatory T (iTreg) cells[41] (Figure 1.1).

Effector cytokine expression and secretion is regulated at several levels[42]: epigenetic changes take place during differentiation that lead to expression or silencing of cytokine genes[43,44,45]; transcription is enhanced or repressed by transcription factors, and post-translational mechanisms regulate mRNA stability or protein secretion. Most of the cytokine regulation is carried out by lineage specific master transcription factors. Cytokines signal in an endocrine manner: receptors on the membrane of target cells relay the cytokine signals intracellularly, often via the STAT protein family. The polarising cytokines, master transcription factors and signature cytokines of the main Th cell types are listed below.

Th1 cells are characterised by a high T-bet expression[46]. The main Th1 cytokine is IFN-γ and contributes to the response against intracellular pathogens by signalling to macrophages. Their differentiation is driven by IFN-γ/STAT1 and IL-12/STAT4.

Th2 cells show a high GATA-3 expression[47]. They secrete mainly IL-4, a cytokine that enhances the antigen-presenting capacity of B cells and fights extracellular parasites, but also IL-5 and IL-13[48]. Their differentiation is driven by IL-4/STAT6.

Th17 cells express the transcription factor RAR-related orphan receptor (ROR)γt[49]. They produce, among others, IL-17A, which activate fibroblasts, epi- and endothelial cells as well as stromal cells and recruit neutrophils. Th17 cells also produce IL-17F, IL-21 and IL-22[50,51]. Their differentiation is driven by Transforming growth factor (TGF)-β/SMAD2/3 and IL-6/STAT3[52].

Treg cells are characterised by a high Forkhead box (Fox)P3 expression[53,54] and are responsible for immune tolerance and tuning of the immune response by repressing other immune cells[55]; they can arise already in the thymus (nTreg cells) or during an immune response (iTreg cells)[56]. Their differentiation is driven by TGF-β/SMAD2/3[57].

Tfh cells are dependent on Bcl-6[58,59] and are critical for B cell activation and germinal centre formation by producing IL-4 and IL-21. Their differentiation is driven by IL-6/STAT3 and IL-21/STAT1/3[60].
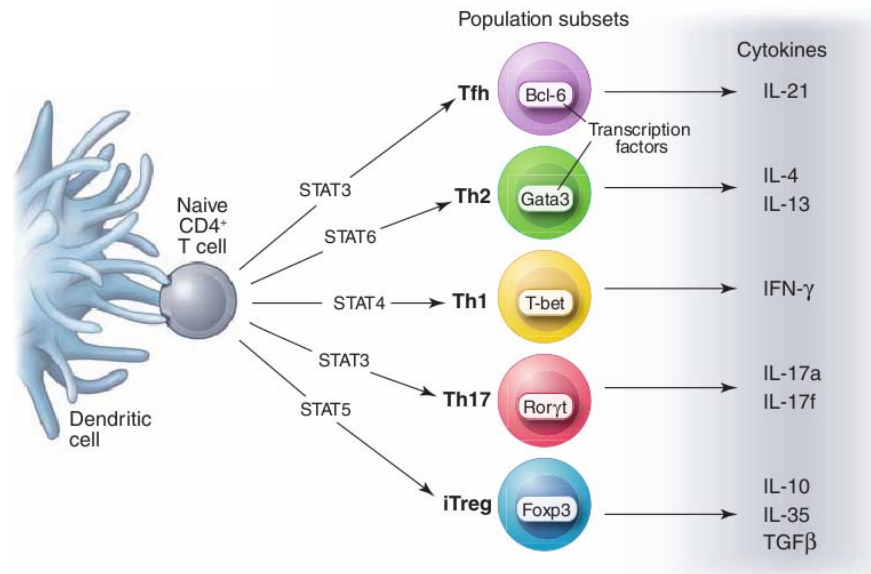
Figure 1.1: Subtypes of CD4+ T cells

Stability and plasticity of Th cell lineages

Antagonistic relationships between the different Th cell lineages have often been
observed. The master transcription factor of Treg cells, Foxp3, is known to
repress the master transcription factor of Th17 cell, ROR t[61]; conversely, the
Th17-polarising cytokine IL-6 represses Foxp3 expression[62]. Foxp3 expression
is also repressed by both T-bet and GATA-3 in Th1 and Th2 cells[63], while IFN-
signalling in Th1 cells and IL-4 signalling in Th2 cells inhibit Th17 differenti-
ation[64,65]. The Th1 and Th2 gene networks contain several mutually repressive
mechanisms discussed in detail below. Those discoveries lead to the belief that T
helper cells make a clear lineage decision during primary activation and cannot
be reprogrammed to express factors belonging to distinct lineages, even though
co-expression of IFN- and IL-4 was observed early after the description of the
Th1 and Th2 lineages[66,67].

In recent years, many discoveries have been made concerning T cell plasticity[68],
showing that differentiated cells can be reprogrammed to co-express factors from
different lineages and challenging the hitherto accepted paradigm of a unique,
lineage-defining transcription factor expression[69]. Virus-specific Th2 cells can
up-regulate both T-bet and IFN- without losing their Th2 characteristics upon
viral infection[70]; similarly, both Th1 and Th17 cells are able to express IL-4 upon

helminth infections[71]. The Th17 phenotype shows particularly high plasticity, as it can acquire Th1 and Th2 features[72,73,74,71,75] as well as be combined to Treg cell characteristics[76,77,78]. The finding that many mutually repressive factors can be stably co-expressed in a single cell still needs to be investigated. The fact that the cells are plastic (i.e., able to adopt some phenotypic characteristic not belonging to their lineage after differentiation) does not mean that they are not stable; indeed, with the exception of Treg cells being converted to pathogenic Th17 cells[79], all other lineages retain the expression of their original master transcription factors and cytokines, although not necessarily in the same amount. This plasticity allows for a better tuning of the immune response and is, in most cases, beneficial.

1.1.4 Th1, Th2 and hybrid Th1/2 cells: co-existence, mutual inhibition and auto-activation

Th1 and Th2 cells are the most abundant subtypes of T helper cells and were the first described T helper phenotypes[80]. Th1 and Th2 cells have distinct activation histories, gene expression profiles and functions. As such, the molecular network underlying their differentiation and maintenance are well-studied, It has long been postulated that the Th1 and Th2 programs were mutually exclusive[81,68], mainly because most studies of Th cells focused on one type of stimulus during differentiation. Furthermore, mutually inhibitory mechanisms between Th1- and Th2-specific factors have been described. However, it has recently been shown that stable cells with a mixed Th1/Th2 phenotype[70,82], expressing both T-bet and GATA-3 as well as Th1 and Th2 cytokines, can be generated both *in vivo* and *in vitro*. This type of cell can be obtained through viral infection of mice after the transfer of virus-specific Th2 cells[70], cells that then start up-regulating T-bet and co-produce IL-4 and IFN-γ while maintaining their GATA-3 expression. T-bet$^+$GATA-3$^+$ cells can also be found in mice after parasite infection with *H. polygyrus*[82]. *In vitro*, T-bet$^+$GATA-3$^+$ cells are obtained when naive cells are activated in the presence of the polarising cytokines IFN-γ, IL-12 and IL-4[82,83,84]. Furthermore, it was shown that a continuum of hybrid states existed between high T-bet expressing Th1 cells and high GATA-3 expressing Th2 cells, depending on the concentration and combination of cytokines present during primary differentiation[83,84].

Th1 cells

Th1 cells mediate type-1 immunity and are defined by the expression of the master transcription factor T-bet[46]. The secretion of IFN-γ as well as high amounts of Tumor necrosis factor (TNF)-α stimulate macrophage, while the expression of the chemokine receptors CXCR3 and CCR5[85,86] leads them to sites of bacterial and
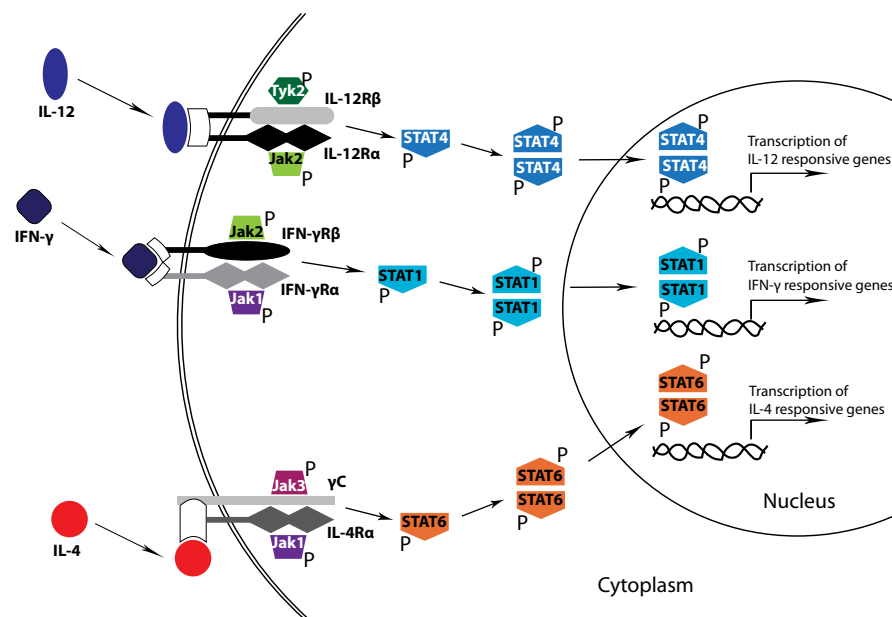
Figure 1.2: Canonical signalling pathways of the cytokines IL-4, IL-12 and IFN-γ.

viral infections where their respective ligands CXCL9 and CXCL10[87], and MIP-1α, MIP-1β and RANTES[88] are expressed[89,90].

The gene network underlying Th1 differentiation and maintenance is not fully known, although several mechanisms are well-described. It involves several positive feedback loops on T-bet and cytokine genes that induce and maintain the Th1 phenotype and the expression of T-bet. Differentiation of naive cells into Th1 cells occurs when naive Th cells are in an IL-4-free environment and in the presence of IL-12[91,92] and IFN-γ[93,94] and happens in several steps. First, IFN-γ, produced by other neighbouring cells or, in the absence of IL-4, stochastically by the naive Th cells upon TCR stimulus, will induce the *Tbx21* gene via STAT1 signalling; T-bet will then up-regulate the expression of both IFN-γ[46] and IL-12Rβ2, which is part of the IL-12 receptor[95]. This renders the cells responsive to IL-12 signalling. Then, external IL-12 can further up-regulate T-bet and IFN-γ in IL-12 responsive cells via STAT4 signalling[96], leading to full Th1 differentiation. Thus, even though T-bet is sufficient to drive IFN-γ expression, Th1 differentiation also relies on both STAT1 and STAT4. Furthermore, T-bet co-operates with several other transcription factors to regulate IFN-γ expression via epigenetic changes at the *Ifng* locus, among which Eomesodermin[97], Runt-related transcription factor (Runx)3[98,97], H2.0-like homeobox protein (Hlx)[99] and E26 transformation-specific (Ets) transcription factors[100,101].

There are thus several cytokine-dependent positive feedback loops on T-bet expression: via IFN-γ signalling, which up-regulates T-bet via STAT1, as well as via IL-12 signalling through the up-regulation of the IL-12 receptor. A more direct feedback loop on T-bet might be achieved through the transcription factor Onecut2, which might be up-regulated by T-bet and might, in turn, enhance T-bet transcription[102], as well as directly by T-bet itself through auto-activation[95,103].

Th2 cells

Th2 cells mediate type-2 immunity and are defined by the high expression of the transcription factor GATA-3[47]. They secrete the signature cytokines IL-4, IL-13 and IL-5 which are involved in class switching of B cells to IgG1 and IgE[104,105] as well as alternative activation of macrophages[106]. The expression of the chemokine receptor CCR4[86] leads them to sites of type-2 inflammation[107].

The Th2 differentiation network also involves positive feedback loops on GATA-3, allowing its long-term expression. Th2 cells arise when IL-4 is present and IFN-γ and IL-12 are absent during primary activation of naive Th cells; IL-4 signalling occurs via STAT6 and up-regulates GATA-3 expression[108,109]. GATA-3 in turn stimulates the expression of IL-4 by the Th2 cell itself[110,111] in concert with IL-2-activated STAT5[108,112,113], as well as its own expression. Other transcription factors are Th2-specific and involved in cytokine regulation; c-Maf and JunB promote IL-4 expression[114,115,116]. Th2 differentiation could also be induced independently of IL-4 through T Cell Factor 1 (TCF-1), which is induced by TCR signalling and activates GATA-3[59]. Thus, as is the case for T-bet in Th1 cells, GATA-3 depends on several other transcription factors to orchestrate full Th2 differentiation, most importantly STAT6.

In this way, GATA-3 expression is amplified in various fashions: it transactivates its gene, thereby directly maintaining its own expression[117,118], and induces IL-4 expression which in turn enhances GATA-3 expression via STAT6[108,109,110,111]. Another feedback loop could be via Dec2 during Th2 differentiation: Dec2 is induced by GATA-3 and induces IL-4, IL-5 and IL-13[48,119,120].

Mutual inhibition of the Th1 and Th2 pathways

The full range of interactions between the two gene networks is still unclear. GATA-3 is known to down-regulate STAT4[121] and IL-12Rβ2[122], which are important parts of the Th1 gene network, and to silence the T-bet locus[123]. Furthermore, GATA-3 has also been reported to silence the IFN-γ locus[124,123] and to block IFN-γ induction by Runx3 and Eomes[97]. c-Maf and TCF-1 are also thought to down-regulates IFN-γ in Th2 cells[114,59]. The transcription factor Ikaros also silences T-bet and IFN-γ in Th2 cells[125,126].

Conversly, T-bet has been reported to bind GATA-3 and repress its transcription factor activity (thereby possibly also its auto-activation)[127]. STAT4 has also been shown to suppress GATA-3 expression, although, as the study predates the discovery of T-bet, it is not clear if the effect is T-bet-mediated or direct[122]. IFN-γ signalling has been linked to a reduced IL-4 expression[128,129]. Another Th1 transcription factor, Runx3, could cooperate with T-bet to silence IL-4 (as well as enhance IFN-γ), and also interacts with GATA-3 to attenuate the Th2 phenotype in Th1 cells[130,98,131]. Lymphoid enhancer-binding factor (LEF)1 is expressed in Th1 but not Th2 cells and seems to down-regulate IL-4 in Th1 cells[132]. Furthermore, TCR-mediated signalling, present during both Th1 and Th2 differentiation, has been reported to bias differentiation depending on its strength and duration: while weak signals were associated with Th2 differentiation, stronger stimulation was linked with Th1 differentiation[133,134,135,136].

Models of Th1 and Th2 regulatory gene networks

Network inference is the reconstruction of biological networks based on large datasets; inference is typically done using high-throughput data. State-of-the-art methods for network inference include machine learning and pattern classification as well as probabilistic methods[137,138,139]. Several studies have used high-throughput data to unravel the network underlying Th cell differentiation, including Th1-Th2 plasticity[140], Th17 differentiation[141,142,143], discovery of master regulators[144], identification of genes regulated by polarising cytokines[145] and involved in Th1/Th2 differentiation[146,147], comparison of Th cell subsets expression profiles[148], epigenetic patterns[149] as well as DNA binding of GATA-3 and STAT proteins[123,150]. The role of STAT6 during Th2 differentiation was studied using RNAi and ChIP sequencing[151]. Network inference allows to describe network topologies that can be used for dynamical modelling.

Dynamical mathematical modelling is a way of describing the dynamics of real phenomena (physical systems, biological interactions, ecological problems or economic situations, for example) by equations representing the different players and their interactions in the modelled system. It can describe exact mechanics and dynamics of a system. Specifically, biological modelling allows to simulate the behaviour of biological systems in order to reproduce experimental data, thereby confirming or excluding mechanisms leading to the observed phenotypes or reconstructing gene regulatory networks. It can also be used to predict the behaviour of a biological system under certain conditions, for example the knockout of genes in a regulatory network, to discover new mechanisms and new roles for these mechanisms.

Several mathematical models of Th cell regulatory networks, and specifically Th1 and Th2 networks, can be found in the literature[152]. Suppression of one

phenotype by the other was present in an early model in the form of competition for antigenic stimulation and cross-suppression by cytokines[153]. Later models model the expression of the master transcription factors T-bet and GATA-3; T-bet expression has been modelled by E. Schulz et al.[96] considering IL-12Rβ2, T-bet and IFN-γ. In this model, TCR signalling activates T-bet, which in turns promote the expression of IFN-γ and IL-12Rβ2, the latest being repressed by TCR signalling at the beginning of differentiation. IFN-γ then enhances T-bet expression via STAT1; later on, IL-12 signalling via STAT4 enhances T-bet expression, but also IFN-γ expression. The crosstalk between IL-12, IFN-γ and TNF-α has been investigated using a Th1 cell model[154]. GATA-3 expression and regulation has been modelled by T. Höfer et al.[155] and L. Mariani et al.[156]. In these models, GATA-3 is induced by STAT6 signalling and trans-activates its own gene; interleukins are not included. In T. Höfer et al.'s model, several processing steps between mRNA and protein are included, whereas L. Mariani et al.'s model uses only one translation step. Both models generate a bistable behaviour for GATA-3 protein expression.

Simple models of interactions between T-bet and GATA-3 (and so the Th1 and Th2 networks) have also been published[157,158]; they include auto-activation loops and mutual repression between GATA-3 and T-bet. In one model, polarising cytokines are included as parameters[157], and are not present in the other model[158]. A complex model aiming at explaining the principal constituents of the regulatory networks in terms of cytokines, cytokine receptors and transcription factors has also been published[159]; however, this model is not data-based. Furthermore, models including other Th cell phenotypes like Treg and Th17 cells have been developed to study Th cell differentiation[160,161,162,163]. All of these models are based on the assumption that T-bet and GATA-3 are mutually exclusive, so that none of them account for co-expression of T-bet and GATA-3 or explain the signal integration process by the cells when several distinct cytokines are present during differentiation, even though one model accounts for Treg/Th17 plasticity[163].

Following the numerous descriptions of Th cell plasticity, models studying this process have been developed. A logical, non-data-based model predicted the four stable Th1, Th2, Th17 and Treg states as well as transient hybrid states[164]. The combination of microarray human gene data and large gene network analysis allowed the development of a gene regulatory network describing a stable Th1/Th2 hybrid state extending beyond T-bet and GATA-3[140]. This model did not, however, explain quantitative differences but only represented the genes as active or inactive. The plasticity of the different Th subsets has been investigated using a logical model, making predictions about possible hybrid cell types not yet described[165].

1.2   Aim of the study

This study focuses on Th1 and Th2 cells, the most abundant T helper cell subtypes, as well as hybrid Th1/2 cells. More precisely, we will focus on the molecular regulation of their differentiation programs.

Dysregulations of the T helper cell immune response are involved in several human diseases. Th1 cells are linked to autoimmune diseases such as type-1 diabetes and rheumatoid arthritis[166,167] and to chronic inflammation[168], while Th2 response imbalance is involved in allergic reactions such as asthma[169] or antibody-linked autoimmune diseases such as lupus[170]. It is therefore important to understand the mechanisms underlying Th cell differentiation, memory, phenotype maintenance as well as the interactions between differentiation programs that allow a well-tuned immune response. Furthermore, Th cells are a well-established model of cell fate decision and lineage commitment in biology, so that understanding their differentiation pathways and maintenance mechanisms may provide a blueprint for understanding many other biological phenomena. Indeed, the concept of master regulators extends beyond the immune system and is pivotal in the study of lineage decision-making (e.g, in stem cell and cancer biology).

The goal of this project is to get an insight into the gene networks underlying Th1, Th2 and hybrid Th1/2 cell differentiation, more precisely the regulation of the master transcription factors T-bet and GATA-3, which govern the functional activity of Th1 and Th2 cells, respectively. Through experimental work and data-based mathematical modelling, the regulatory mechanisms leading to T-bet and GATA-3 expression patterns will be analysed in order to answer the following questions:

- Can a methodology be developed to reconstruct gene regulatory networks based on straightforward experiments and basic knowledge of the network's hierarchy?

- How does a cell integrate several differentiating signals to decide its phenotype?

- How do the Th1 and the Th2 lineage-specific and signature genes influence each other during differentiation?

- What determines the amplitude of the functional response in differentiated cells?

1.3   Methodology

In this section, we present the general experimental and computational approach we used to address the questions at the centre of this study. Detailed protocols are
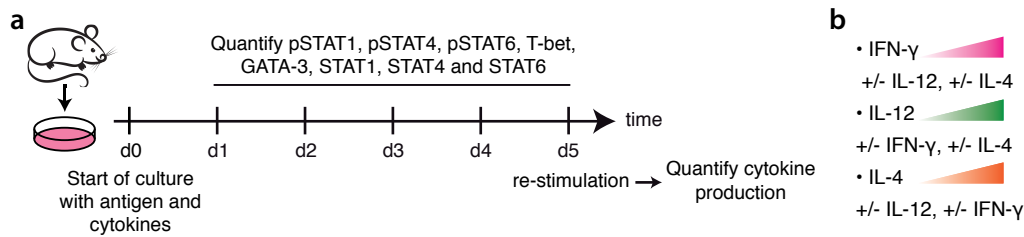
Figure 1.3: Experimental protocol. a Freshly *ex vivo* isolated naive CD4+ T cells were cultured for five days with antigen presenting cells, antigen, IL-2, polarising cytokines and blocking antibodies. pSTATs, total STATs, T-bet and GATA-3 were measured by flow cytometry on a daily basis, and the cytokine production after restimulation was quantified on day 5. **b** The three main Th1 and Th2 skewing cytokines IFN-, IL-12 and IL-4 were titrated in naive CD4 T cell cultures in the absence and/or presence of the other two cytokines.

described in the Materials and Methods section. We used a combination of highly quantitative experiments, linear regression analysis and dynamical modelling to unravel the cytokine network underlying Th1 and Th2 differentiation.

Experimental setup

To obtain an integrated picture of signalling and gene expression during Th1 and Th2 cell differentiation, we differentiated freshly *ex vivo* isolated, FACSorted naive CD4$^+$ LCMV-TCR^{tg} T cells in the presence of cognate antigen stimulus (in form of GP64$_{(61\ 80)}$ peptide), the growth factor IL-2 and the instructive cytokines IFN-, IL-12 and/or IL-4 (Figure 1.3). We call cells generated with IFN- and/or IL-12 and anti-IL-4 'Th1'; cells generated with IFN- and/or IL-12 and IL-4 'hybrid Th1/2' and cells generated with anti-IFN-, anti-IL-12 and IL-4 'Th2'. We measured the phosphorylation levels of the three main signal transducers downstream of the instructive cytokines (STAT1, STAT4 and STAT6) as well as their total protein levels and those of the master transcription factors T-bet and GATA-3 by flow cytometry on a daily basis up to day 5 of differentiation. To characterise the functional phenotype of the cells after differentiation, we re-stimulated the cells in the absence of instructive cytokines with PMA/ionomycin, mimicking a strong antigen stimulus, and measured the production of key cytokines by flow cytometry after intracellular cytokine staining. We thus obtained quantitative information on signal transduction and transcription factor expression from early to late differentiation as well as a functional read-out in differentiated cells.

To investigate how the cells respond to a broad range of mixed cytokine stimuli, in particular how different combinations and amounts of IFN-, IL-12 and IL-4 influence master transcription factor expression, we titrated each one of the three cytokines (e.g., IFN-) in the presence or absence of one or both of the other cytokines (e.g., IL-12, IL-4) (Figure 1.3b). The titration steps were

chosen empirically as to induce different levels of T-bet and/or GATA-3 upon differentiation. This resulted in a total of twelve groups of titrations, with 48 different cytokine combinations each evaluated at five time points (a list of all conditions used is available in Supplementary Table 7.1). Th cells do not produce IL-12, but may produce IFN-γ or IL-12; to ensure that the amounts of polarising cytokines in the culture medium were strictly controlled and that the results were not confounded by IFN-γ or IL-4 produced by the Th cells themselves during the culture, we used *Ifng-/-* T cells as well as blocking antibodies to IL-4 in the Th1 conditions for the IFN-γ and IL-12 titrations. Similarly, we used *Il4-/-* cells and blocking antibodies to IFN-γ in the Th2 conditions for the IL-4 titrations. Furthermore, we used cells deficient in both IL-12 and IFN-γ as antigen-presenting cells (no significant amount of IL-4 is produced by the APCs used in this setup). Thus, the levels of polarising cytokines are determined externally.

The data generated with these time-resolved titration experiments was analysed to reconstruct the regulatory network through which master transcription factors are regulated by the STATs and each other, but also through which the STATs themselves and cytokine expression are regulated.

Computational analysis

The experimental part of this project generated a large amount of data: eight proteins were measured at five different time points in 48 different conditions. Although a first visual analysis of the data put several mechanisms in evidence, it soon became clear that the number of regulatory interactions involved made an unambiguous interpretation of the origin of the observed effects difficult. To systematically analyse this titration data and derive a general topology of the signalling network underlying Th1 and Th2 differentiation, we used a series of linear regression models describing how a layer of regulators (e.g., cytokines or pSTATs) controls a corresponding layer of regulatees (e.g., pSTATs or transcription factors, respectively):

$$\text{regulatee}_i = \alpha_0 + \sum_j \alpha_j \cdot \text{regulator}_j$$

with all combinations of j for each i.

We thus obtained families of linear models describing the amount of each protein quantified during the titration experiments in function of the polarising cytokines used during the cell cultures. Model comparison using goodness of fit measures (R^2 and Akaike information criterion (AIC)) quantified the correlations between the factors measured. Furthermore, we used the time-resolved data to gain information about the kinetics of regulation by taking the amount of regulators at

previous time points into account to explain the amount of regulators at a given moment, i.e., a weighted sum of the expression of the regulators over time was used to explain the expression of the regulatee at each time point. Repetition of this analysis for each time point and each layer considered, i.e., STAT phosphorylation by the cytokines, transcription factors expression regulation by the activated STATs and the master transcription factors, and cytokine expression regulation by T-bet and GATA-3 allowed us to determine the most probable complete network of interactions at each time point.

Linear models can describe the general topology of a network, but usually cannot capture its dynamics. Furthermore, a linear model allows only linear interactions between one layer of regulators and one of regulatees, and thus cannot describe feedback loops such as auto-activation. To further study those points and test the network topology against time-resolved data, we designed a dynamical, ordinary-differential-equations (ODE)-based model derived from the linear-regression network topology. This model was used to reproduce the kinetics of phosphorylation and/or up-regulation of the transcription factors STAT1, STAT4, STAT6, T-bet and GATA-3, to predict the response of the system to gradients of polarising cytokines, and to study the relative effect of the phosphorylated STAT and MTF proteins on the final phenotype of the Th cells.

Th1 and Th2 cells are the most abundant subtypes among T helper cells, which are part of cellular adaptive immunity and are responsible for coordinating the adaptive immune response via cytokine secretion. Th1 cells are characterised by the expression of the master transcription factor T-bet, which is up-regulated by IFN-γ/STAT1 and IL-12/STAT4 signalling and leads to IFN-γ secretion, while Th2 cells are characterised by high expression of the master transcription factor GATA-3, which is up-regulated by IL-4/STAT6 signalling and leads to IL-4 secretion. Although the Th1 and Th2 lineages have long been thought to exclude one another due to several mutually repressive mechanisms involving their master transcription factors, hybrid Th1/2 cells have recently been shown to arise and remain stable *in vivo*. This works aims at understanding how mixed cytokine signals are integrated during Th cell differentiation and how they influence master transcription factor expression and lineage decision, using quantitative time-resolved experiments followed by computational analysis in form of linear regression and dynamical mathematical modelling.
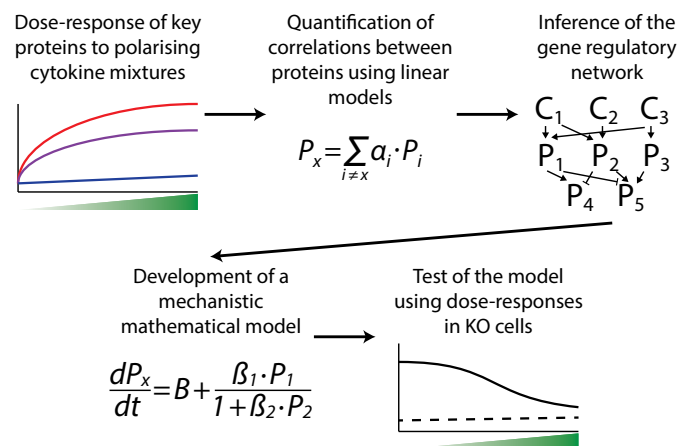

Figure 1.4: Methodology.

Chapter 2

Experimental results

Th1 differentiation occurs when IFN-γ and IL-12 up-regulate T-bet expression via STAT1 and STAT4 signalling, respectively[93,94,91,92], while Th2 differentiation requires GATA-3 up-regulation following STAT6 signalling[108,109]. Hybrid Th1/2 cells arise when all three signalling pathways are activated simultaneously, leading to the up-regulation of T-bet and GATA-3[82,83,84]. Both Th1 and Th2 differentiation have been studied extensively under conditions which elicit one or the other response. However, the molecular network that mediates the responses of naive T helper cells to gradients and combinations of cytokines remains poorly understood. This chapter describes the qualitative changes occurring during differentiation in response to distinct cytokine amounts and combinations at three levels (Figure 2.1): the phosphorylation of the STATs, the protein expression of T-bet, GATA-3, STAT1, STAT4 and STAT6, and the cytokine production by the differentiated Th cells upon restimulation. We start by studying the kinetics of expression and phosphorylation in 'classical' conditions, i.e., Th1, Th2 and hybrid Th1/2 with fixed cytokine concentrations, in order to determine how the polarising cytokines used in standard *in vitro* setups affect the expression and activity of various regulators. We then go on to qualitatively analyse the dose-response of the transcription factors to the cytokines IFN-γ, IL-12 and IL-4 and their response to different cytokine combinations.

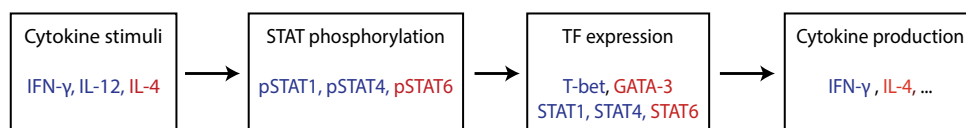| Cytokine stimuli	STAT phosphorylation	TF expression	Cytokine production
IFN-γ, IL-12, IL-4	pSTAT1, pSTAT4, pSTAT6	T-bet, GATA-3 STAT1, STAT4, STAT6	IFN-γ , IL-4, ...

Figure 2.1: Sequential levels of response to cytokine stimuli during differentiation.
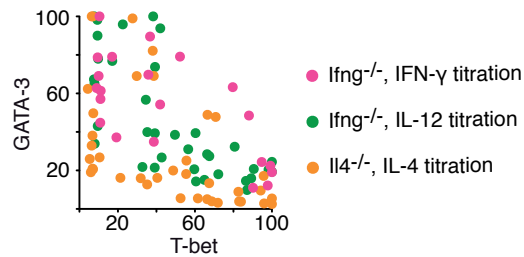
Figure 2.2: Quantitative differences in cytokine concentrations are reflected on the MTF expression.
A continuum of T-bet-GATA-3 co-expression patterns was generated on day 4 in response to the titrated
cytokines, normalised geometric mean indices for all conditions are shown.

2.1 STAT, master transcription factor and cytokine expressions are dynamically regulated in Th1, Th2 and Th1/2 hybrid cells

Previous work has shown that Th1 versus Th2 differentiation is driven by the
cytokines IL-12[91,92] and IFN-γ[93,94] versus IL-4[108,109], acting through their canonical
Jak-STAT pathways (STAT1 for IFN-γ[171,172], STAT4 for IL-12[173,174], and STAT6 for
IL-4[175,176]) to up-regulate T-bet versus GATA-3; these studies have focused on
either Th1-polarising or Th2-polarising stimuli. However, T-bet and GATA-3 were
recently shown to be co-expressed in a gradual manner in response to increasing
amounts of cytokines[83,84]. This co-expression was accompanied by the capacity
to express both Th1 and Th2 cytokines as well as the simultaneous activation of
STAT1, STAT4 and STAT6[82]. Furthermore, quantification of T-bet and GATA-3
protein amounts after differentiation showed that Th cells can adopt a continuum
of T-bet and GATA-3 co-expression levels after primary differentiation, and that
most cytokine combinations actually generated hybrid Th1/2 cells with T-bet and
GATA-3 co-expression (Figure 2.2 and Reference 83). These findings raised the
question of how differences in amounts of polarising cytokines are integrated by a
T helper cell so that they are reflected in the expression of the master transcription
factors and signature cytokines.

We reasoned that for quantitative differences in cytokine amounts to be reflec-
ted on the transcription factor expression level, the strength and/or duration of
signalling downstream of the cytokines must also vary depending on the input
stimuli, which motivated us to study the relation between the amount of phos-
phorylated STAT and master transcription factor. As a matter of fact, we observed
that, for a definite cytokine stimulus, the amount of active signal transducers cor-
related to the amount of target master transcription factor (Figure 2.3): co-staining
pSTAT4 and T-bet or pSTAT6 and GATA-3 in Th1 cells differentiated for 72 hours
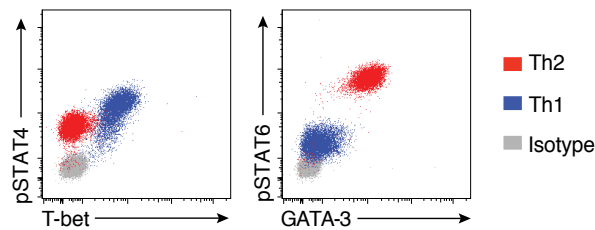
Figure 2.3: Quantitative differences in MTF expression correlates to the amounts of pSTAT. T-bet and pSTAT4, and GATA-3 and pSTAT6 were co-stained in Th1 and Th2 cells after 72 hours of differentiation.

with IL-12 and IFN-γ and in Th2 cells differentiated for 72 hours with IL-4 showed that the cells with the highest master transcription factor expression also had the highest corresponding pSTAT amounts. The stainings for pSTAT4 in Th2 cells and pSTAT6 in Th1 cells can be interpreted as negative even though they are higher than the isotype control; this is a technical artefact due to the staining protocol. Indeed, in order to obtain a good co-staining of the master transcription factors and the pSTAT proteins, BD Phosflow buffers were used as in a total STAT/pSTAT co-staining (see subsection 6.1.2 on page 111), but the last washing step after staining was omitted, causing an increase in pSTAT intensity compared to the standard staining method using which the isotype control was calibrated.

We thus proceeded to quantify the phosphorylation and up-regulation kinetics of the main players in the known signalling cascades involved in Th1 and Th2 differentiation: pSTAT1, pSTAT4 and pSTAT6, T-bet and GATA-3, as well as the total STAT1, STAT4 and STAT6 proteins, in order to see if and how different cytokine combinations affected the amount and dynamics of those transcription factors. Given that T-bet and GATA-3 orchestrate the functional Th cell response, we also measured the production of effector cytokines after TCR restimulation at the end of differentiation. Kinetics and representative flow cytometry stainings in Th1, Th2 and Th1/2 hybrid cells are shown in Figure 2.4.

The phosphorylated STATs constitute the first level of the intracellular response we quantified. The canonical STATs downstream of the polarising cytokines, i.e., STAT1 downstream of IFN-γ, STAT4 downstream of IL-12 and STAT6 downstream of IL-4, were phosphorylated with a unimodal distribution in the cell population; the left panel of Figure 2.4a shows the phosphorylation on day 2 of culture (day 2 was chosen due to the fact that Th cells are responsive to all three cytokine at this time point). This phosphorylation pattern allowed us to use the geometric mean index (GMI, see subsection 6.2.1 on page 112 for a description of the normalisation procedure) of the staining, a population average, for further quantitative analyses. Phosphorylation lasted long with still marked amount of pSTAT4 and pSTAT6 on

day 5. Maximal phosphorylation levels were reached on day 3 for pSTAT1 and pSTAT6 and on day 4 for pSTAT4 (Figure 2.4a, right panel).

The master transcription factors T-bet and GATA-3 were expressed with a unimodal distribution within the Th1, Th2 and hybrid Th1/2 populations (Figure 2.4b, left panel), allowing the use of the geometric mean index for further analysis. Up-regulation started already at day 2 of differentiation, before extensive cell division occurred; the up-regulation of transcription was visible even earlier (day 1; Supplementary Figure 7.3a,b). The combination of IL-4, IL-12 and IFN-γ gave rise to hybrid Th1/2 cells, with co-expression of T-bet and GATA-3 in individual cells albeit at somewhat lower levels than in Th1 and Th2 cells, respectively (shown in purple in Figure 2.4b and from here on), rather than distinct Th1 (shown in blue) or Th2 (shown in red) phenotypes. The simultaneous presence of Th1 and Th2 cytokines affected the expression level of the master transcription factors, but not the kinetics of their up-regulation.

We found that the expression levels of all three STATs were also dynamically and differentially regulated depending on the cytokine milieu; indeed, clear differences in the expression levels could be observed already on day 1 for STAT1 and on day 2 for STAT4 and STAT6. STAT1 and STAT4 were up-regulated in Th1 and hybrid Th1/2 cells whereas STAT6 expression was augmented in Th2 and hybrid Th1/2 cells (Figure 2.4c); these changes were also seen at the RNA level for STAT1 and STAT4 (Supplementary Figure 7.3c,d). The unimodal expression of those five transcription factors allowed us to use the geometric mean index of the stainings for the quantification of their expression.

In contrast to the uniform regulation of transcription factor expression, the cytokine recall response showed the typical separation of the population into responders and non-responders[177,178], with a fraction of hybrid Th1/2 cells producing both IFN-γ and IL-4 (Figure 2.4d). Therefore, we quantified cytokine production using the fraction of producing cells and not the geometric mean.

The observed prolonged phosphorylation of the STATs as well as the changes in their expression levels point to an underlying dynamic regulatory network that evolves on a timescale of days, stimulating a careful kinetic analysis of the changes occurring during differentiation. In the next sections, we thus study the dose response of the pSTATs and transcription factors amounts to cytokines in a time-resolved manner.

STAT1, STAT4 and STAT6 were phosphorylated with a unimodal distribution during up to five days in the presence of their canonical cytokines IFN-γ, IL-12 and IL-4, respectively. The expression of the master transcription factors and the STATs was dynamically and differentially regulated during Th1, Th2 and hybrid Th1/2 differentiation. The Th1 factors T-bet, STAT1 and STAT4 were highly expressed in Th1 cell, intermediate in hybrid Th1/2 cells and low in Th2 cells. The Th2 factors GATA-3 and STAT6 showed the inverse behavior.
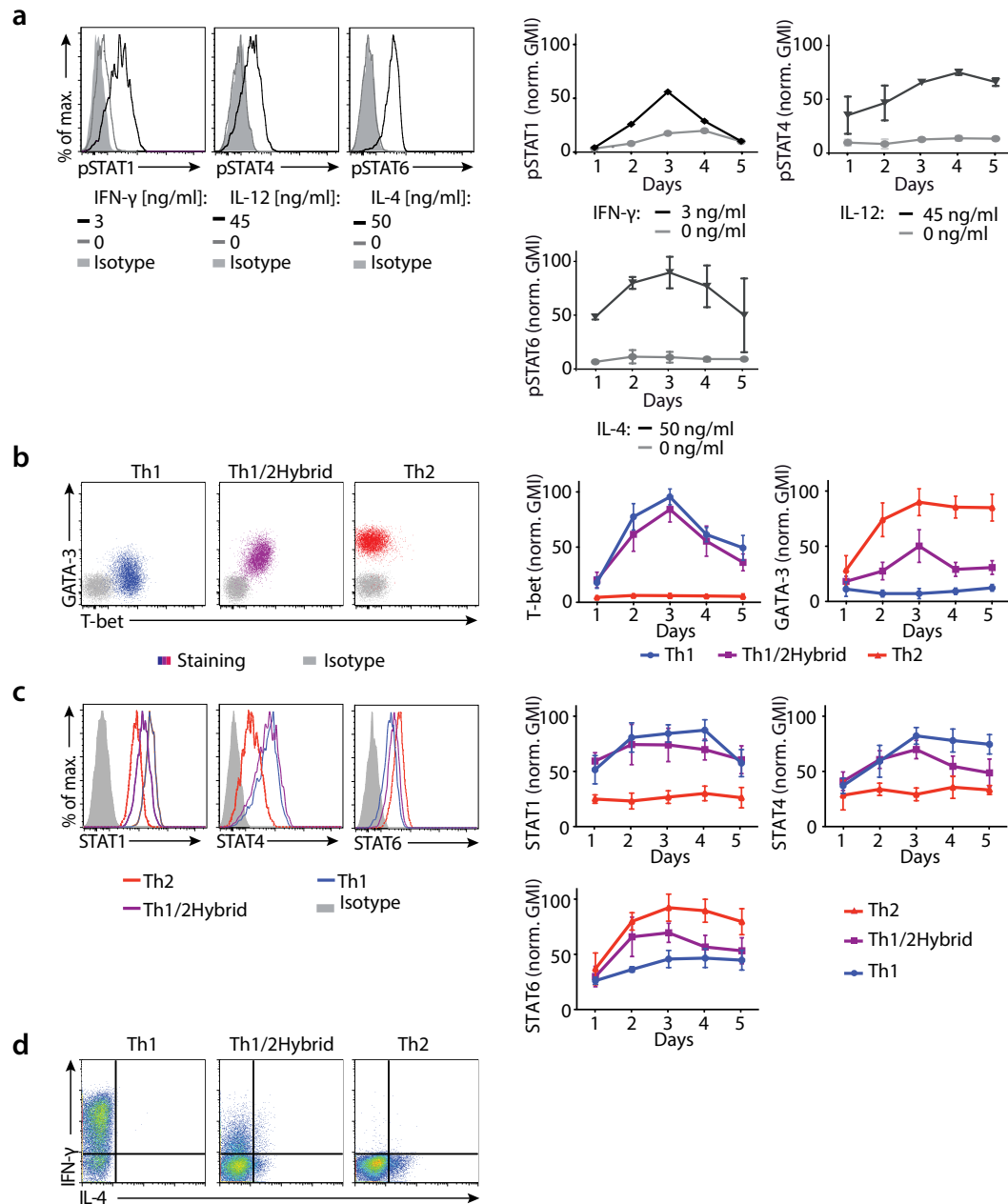
Figure 2.4: pSTATs, MTFs and STATs are up-regulated in a unimodal and cytokine-specific manner during primary differentiation. a pSTAT1, pSTAT4 and pSTAT6 were measured on a daily basis by flow cytometry. Left panel: left: pSTAT1 in response to IFN-γ on day 2; middle: pSTAT4 in response to IL-12 on day 2, right: pSTAT6 in response to IL-4 on day 2. Right panel: left: pSTAT1 in response to IFN-γ (n=1); middle: pSTAT4 in response to IL-12 (n=2), right: pSTAT6 in response to IL-4 (n=2). **b-d** Flow cytometry measurements of T-bet, GATA-3, STAT1, STAT4, STAT6, IL-4 and IFN-γ in Th1, hybrid Th1/2 and Th2 cells. **b** T-bet and GATA-3 flow cytometry measurements on day 4 of culture (left panel) and during the whole week (right panel) in Th1, hybrid Th1/2 and Th2 cells (n=5). **c** STAT1, STAT4 and STAT6 flow cytometry measurements on day 4 of culture (left panel) and during the whole week (right panel) in Th1, hybrid Th1/2 and Th2 cells (n=5). **d** IFN-γ and IL-4 flow cytometry measurements on day 5 of culture after PMA/ionomycin restimulation in Th1, hybrid Th1/2 and Th2 cells.

2.2 STAT activation is dose-dependent, long-lasting and happens outside of the canonical pathways

In the previous section, we showed that STAT4 phosphorylation correlated positively with T-bet expression, and STAT6 phosphorylation with GATA-3 expression, at the single cell level. Furthermore, a unimodal and long-lasting phosphorylation of STAT1, STAT4 and STAT6 in response to IFN-γ, IL-12 and IL-4, respectively, was observed. In order to understand how adverse signals, like IFN-γ and IL-4 or IL-12 and IL-4, are integrated by the the cells during hybrid Th1/2 differentiation as well as how quantitative differences in cytokine concentrations are translated into graded master transcription factor expression, we asked how the presence of multiple and particularly mixed Th1-Th2 stimuli affects the way cytokines act on each STAT pathway. To this end, we measured the phosphorylation of the three relevant STATs during a 5-day time course in *ex vivo* isolated naive murine CD4 T cells, carrying an LCMV-specific T cell receptor, in the presence of antigen-presenting cells loaded with cognate antigen and different mixtures of cytokines to induce differentiation into Th1, Th2 or hybrid cells. We titrated each cytokine (e.g., IFN-γ) in the presence and absence of one or both of the other cytokines (e.g., IL-12, IL-4) and examined the response of each STAT to all three cytokines. Although we focused on the dose-response of the canonical pSTAT downstream of each cytokine in the following sections, all three pSTATs were measured and analysed during each titration experiment; the data is not shown here to avoid unnecessary redundancy.

We start by analysing STAT1 phosphorylation in response to an IFN-γ titration, as IFN-γ induces the phosphorylation of STAT1[171,172]. To examine the response of pSTAT1 to different doses of IFN-γ in combination with other cytokines, we performed a 5-step titration of IFN-γ (i.e., no IFN-γ, 0.003, 0.03, 0.3 and 3 ng/ml of IFN-γ) in the presence of 5 ng/ml IL-12, 10 ng/ml IL-4, both of them, and none of them. Representative flow cytometry stainings for selected conditions on day 2 and the whole normalised dataset for pSTAT1 are shown in the form of GMI time courses in Figure 2.5. The different IFN-γ concentrations led to graded pSTAT1 elevations in both Th1 and hybrid conditions, but did not influence the duration of phosphorylation (Figure 2.5): no pSTAT1 was detected on day 5 in Th1 cells, no matter what the initial IFN-γ concentration was, and little in hybrid cells. Little effect of the two smaller concentrations could be observed, with only a small increase compared to the condition without IFN-γ on days 3 and 4. Furthermore, IL-12 and IL-4 also influenced STAT1 phosphorylation. Indeed, comparing the left and right columns in Figure 2.5b, we see an increase in pSTAT1 in the presence of IL-12. Interestingly, the presence of the Th2 cytokine IL-4 did not reduce the amount of pSTAT1; on the contrary, the latter seemed to increase in
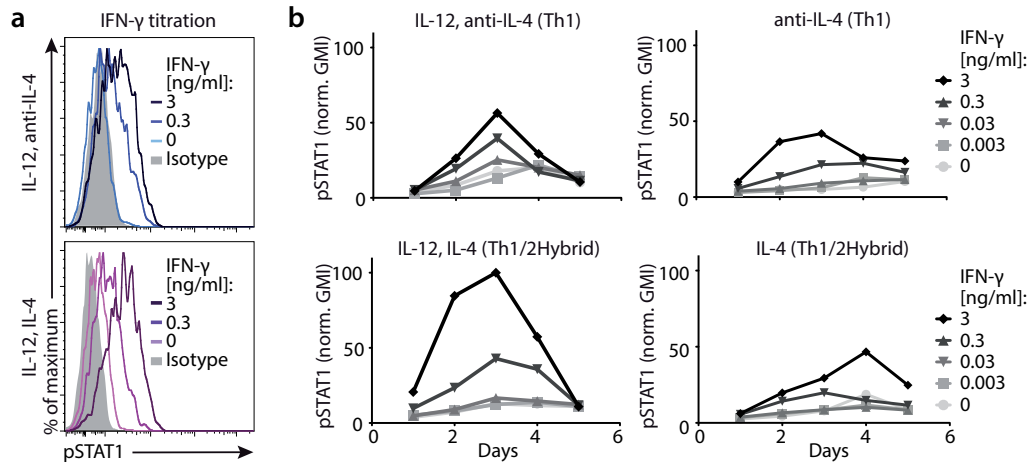
Figure 2.5: pSTAT1 is phosphorylated gradually in response to titrated IFN-γ. a pSTAT1 stainings on day 2 of culture with increasing amount of IFN-γ. **b** Time courses of STAT1 phosphorylation with increasing amounts of IFN-γ with IL-12 (left) or without IL-12 (right), anti-IL-4 (top) or IL-4 (bottom).

the presence of IL-4 (compare top and bottom rows in Figure 2.5b and the positive staining without addition of IFN-γ in the lower panel of Figure 2.5a). Both those effects could also be observed by measuring pSTAT1 in response to IL-12 and IL-4 titrations.

To study how STAT4 phosphorylation was induced by IL-12[173,174] and other cytokines, we performed a 4-step titration of IL-12 (i.e., no IL-12, 0.5, 1.5 and 45 ng/ml IL-12) in the presence of 10 ng/ml IFN-γ, 10 ng/ml IL-4, both and none of them, similarly to what was done with IFN-γ. Representative flow cytometry stainings on day 2 and the whole dataset for pSTAT4 in form of GMI time courses are shown in Figure 2.6. Similarly to STAT1 downstream of IFN-γ, the amount of pSTAT4 per cell increased gradually in response to IL-12, most notably so from day 3 on; indeed, the differences in pSTAT4 intensity were small until the peak of phosphorylation was reached. However, the period during which STAT4 was phosphorylated also depended on IL-12: the more IL-12, the longer pSTAT4 could be detected by flow cytometry: no phosphorylation was measured on day 5 with the smaller IL-12 concentrations, while its level were nearly equal to the peak levels with the highest concentration in Th1 conditions. Comparing the left and right columns in Figure 2.6b, we see that the pSTAT4 levels are globally lower in the absence of IFN-γ. IL-4 seemed to have two distinct effects: it increased the intensity at the peak phosphorylation on day 3, but decreased it on later days (compare upper and lower panels).

STAT6 is known to be phosphorylated downstream of IL-4[175,176]. We studied its response to IL-4, IL-12 and IFN-γ using the same protocol used for pSTAT1 and pSTAT4: we titrated IL-4 in 4 steps (no IL-4, 0.5, 5 and 50 ng/ml IL-4) in the

Figure 2.6: pSTAT4 is phosphorylated gradually in response to titrated IL-12. a pSTAT4 stainings on day 2 of culture with increasing amount of IL-12. **b** Time courses of STAT4 phosphorylation with increasing amounts of IL-12 with IFN-γ (left) or without IFN-γ (right), anti-IL-4 (top) or IL-4 (bottom).

presence and absence of 5 ng/ml IL-12 and/or 10 ng/ml IFN-γ and quantified pSTAT6 every day. Representative data from day 2 and the kinetics of pSTAT6 GMI in all the conditions are shown in Figure 2.7. STAT6 was phosphorylated in a graded manner in response to IL-4 in all the conditions observed, with clear differences visible from day 1 up to day 5. As is the case for pSTAT4 downstream of IL-12, phosphorylation of pSTAT6 could still be measured on day 5 when high IL-4 concentrations were used. The presence of IL-12 and IFN-γ did not induce significant changes in the pSTAT6 intensities, but peak phosphorylation was reached earlier, on day 2 rather than day 3, in the presence of IL-12 (compare upper and lower rows of Figure 2.7b).

> The amplitude of the pSTAT signals increased with the concentration of the corresponding canonical cytokines. However, the presence of other cytokines also affected signalling: IL-4 increased the peak levels of pSTAT1 and pSTAT4 in response to IFN-γ and IL-12, but also caused lower pSTAT4 on day 4 and 5. IL-12 augmented the pSTAT1 levels in addition to increasing the pSTAT4 levels. Importantly, this data indicates that cytokines additional to the canonical ones induce and modulate STAT phosphorylation.
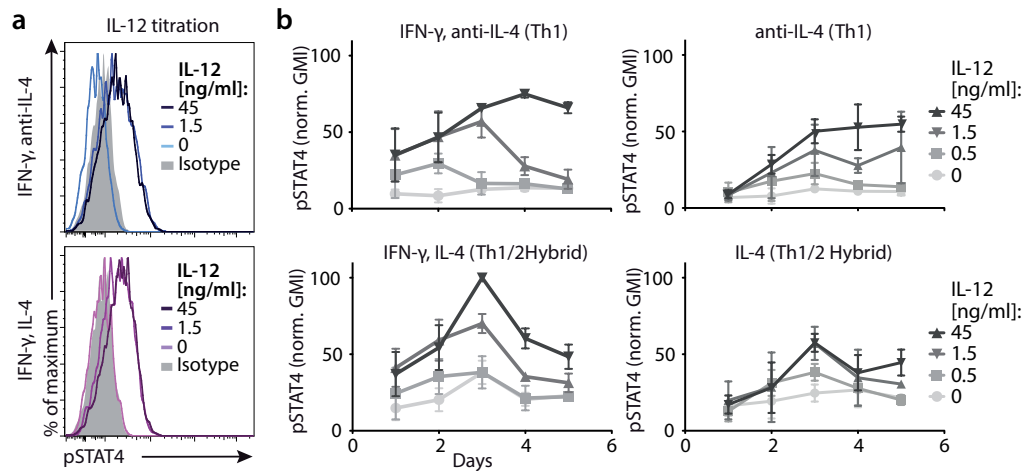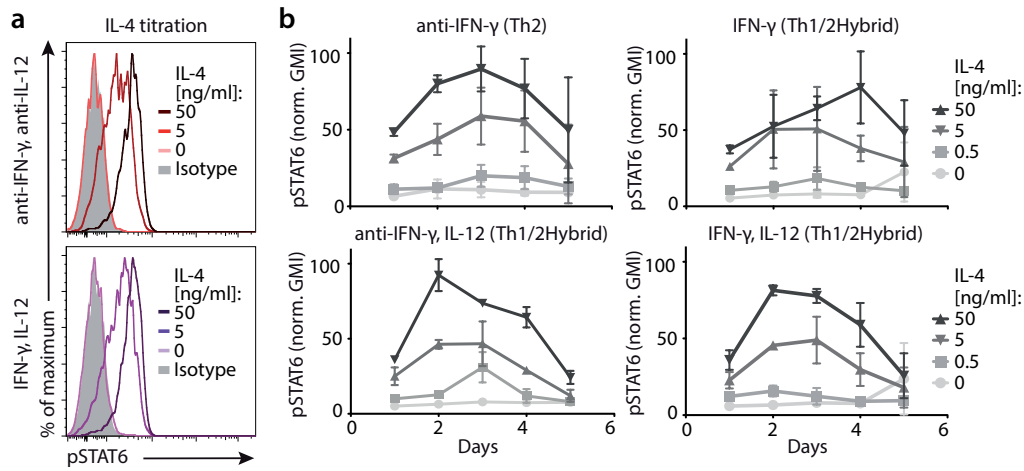
Figure 2.7: pSTAT6 is phosphorylated gradually in response to titrated IL-4. a pSTAT6 stainings on day 2 of culture with increasing amount of IL-4. **b** Time courses of STAT6 phosphorylation with increasing amounts of IL-4 with IL-12 (bottom) or without IL-12 (top), IFN-γ (right) or anti-FN-γ (left).

2.3 Transcription factor expression is regulated both positively and negatively in a dose-dependent manner downstream of polarising cytokines

We focus on five of the transcription factors involved in Th cell differentiation and function that are critical for both lineage decision and cytokine expression regulation. The master transcription factors T-bet (expressed in Th1 cells[46]) and GATA-3 (highly expressed in Th2 cells[47]) are both necessary and sufficient for determining the gene expression profile of differentiated Th cells. STAT proteins, in particular STAT1, STAT4 and STAT6, are essential parts of the signalling pathways controlling T-bet and GATA-3 expression[40]. In the previous sections, we showed that all five proteins were differentially regulated in Th1, Th2 and hybrid Th1/2 cells during primary differentiation and that the upstream pSTAT phosphorylation was dose-dependent and influenced by cytokines within and outside of their canonical pathways. Here, we concentrate on the effect the same cytokines exert on the expression levels of those five transcription factors using titrations as described previously.

2.3.1 T-bet and GATA-3 can be regulated in a synergistic manner by several cytokines and do not respond differently to antigen concentration

Key targets of pSTAT1/pSTAT4 and pSTAT6 downstream of IFN-γ/IL-12 and IL-4 are the master transcription factors T-bet and GATA-3, respectively[93,94,91,92,108,109]. The latter are up-regulated in differentiating Th cells under Th1 conditions (T-bet), Th2 conditions (GATA-3) and hybrid Th1/2 conditions (T-bet and GATA-3[82,83,84]) and direct the expression of other lineage-specific genes that are essential for T cell function, most notably cytokines and chemokine receptors. In addition to cytokine signalling via activated STATs, TCR signalling in response to antigen is necessary for the induction of the master transcription factors in naive cells. In the next sections, we analyse the response of master transcription factors to antigen stimulus and cytokine signals.

Antigen concentration does not bias Th cell differentiation

Previous studies have shown that the strength of the TCR stimulus influences Th1 or Th2 cell fate decision[133,134,135,136], but how universal these findings are is unclear. To investigate how our system responded to titrated antigenic peptides, we performed a 5-step titration of the lymphocytic choriomeningitis (LCMV)-derived GP64-peptide (i.e., 0.05, 0.2, 0.8, 3.2 and 12.8 μg/ml GP64-peptide), for which the transgenic cells used throughout this study have a specific TCR, in IFN-γ-, IL-4-competent cells (Figure 2.8a). In order to discriminate between potentially confounding effects of antigen signalling and cytokine signals and detect eventual synergetic effects of antigen and cytokine signalling, the titration was performed under several differentiating conditions: the 'antigen only' condition, without addition of any cytokine or blocking antibody; the 'Th0' condition, in which the three major cytokines IFN-γ, IL-12 and IL-4 were blocked, as well as 'classical' Th1 and Th2 conditions and hybrid Th1/2 conditions. The cytokine IFN-γ was not added to the polarising mix, but produced by the T cell themselves. The flow cytometry quantification of T-bet and GATA-3 after four days of differentiation is shown in figure 2.8; similar results were obtained at day 2 and 6 of differentiation.

We observed no significant bias towards Th1 or Th2 differentiation depending on the peptide concentration. The amount of peptide showed no specific correlation with the mean per cell expression of T-bet or GATA-3 during differentiation. Overall, the cells seemed to express higher amounts of master transcription factor with higher antigen doses in the conditions where autogenous cytokine signalling was involved: T-bet increased with the GP64 concentration in Th1, hybrid Th1/2 and antigen only conditions, all conditions in which Th cells produced IFN-γ; the same was true for GATA-3 in Th2 and hybrid Th1/2 conditions in which IL-4 is

produced by the cells (Figure 2.8b). This suggests that a higher antigen concentration increases the whole activation status of the cells, thereby increasing their cytokine production during the early days of differentiation, without specifically influencing lineage decisions. However, higher antigen concentrations seemed to repress the emergence of a population expressing intermediate levels of GATA-3 among the otherwise unimodal T-bet-positive Th1 cells, and to induce a small T-bet-positive population in GATA-3-positive Th2 cells. The bimodality observed here under Th1 and Th2 conditions in IFN-γ-, IL-4-competent cells was not seen in experiments using *Ifng-/-* cells under Th2 conditions or *Il4-/-* cells under Th1 conditions. We thus did not consider this phenomenon further, as it is probably due to the imperfect blockade of stochastically produced cytokines.

IFN-γ and IL-12 lead to the dose-dependent up-regulation of T-bet and repression of GATA-3, while IL-4 correlates positively with GATA-3

Having established that antigen concentration does not bias cell differentiation in either the Th1 or Th2 direction in our system, we proceeded to study the effects of the polarising cytokines. Previous studies[83,84] and unpublished work by M. Peine and C. Helmstetter shown in Figure 2.9 have shown that T-bet and GATA-3 can have a continuum of co-expression patterns if the Th cells are differentiated with varying amounts of cytokines. To quantify the effect of each cytokine on each master transcription factor, we used the same titration experiments during which STAT phosphorylation was measured, i.e., IFN-γ, IL-12 and IL-4 were titrated separately in the presence of one, both and none of the other two cytokines. T-bet and GATA-3 were quantified on a daily basis during the 5-day differentiation. We then analysed how multiple cytokine stimuli govern the expression of the master transcription factors T-bet and GATA-3 in the differentiating (days 1-4) and differentiated (day 5) cells. To be specific, we show here the data for day 4 of culture as dose-response curves of master transcription factor expression versus cytokine concentration; previous days and day 5 all gave qualitatively similar results; day 4 represents a time-point at which differentiation is nearly completed and the cells are still activated.

First focusing on the response of the Th1 master transcription factor T-bet to IFN-γ (Figure 2.10a), we observed a graded up-regulation of T-bet protein in response to IFN-γ in most conditions. However, when the Th cells were differentiated in the presence of the Th1-polarising cytokine IL-12 in the absence of the Th2-polarising cytokine IL-4 (full blue line), the addition of IFN-γ did not further increase the expression of T-bet. Contrastingly, when IL-4 was added to the culture (full purple line), the already up-regulated levels of T-bet rose in a dose-dependent manner in response to IFN-γ in a steeper manner than in the absence of IL-12, reaching nearly saturation and Th1-levels for the highest concentrations. In the
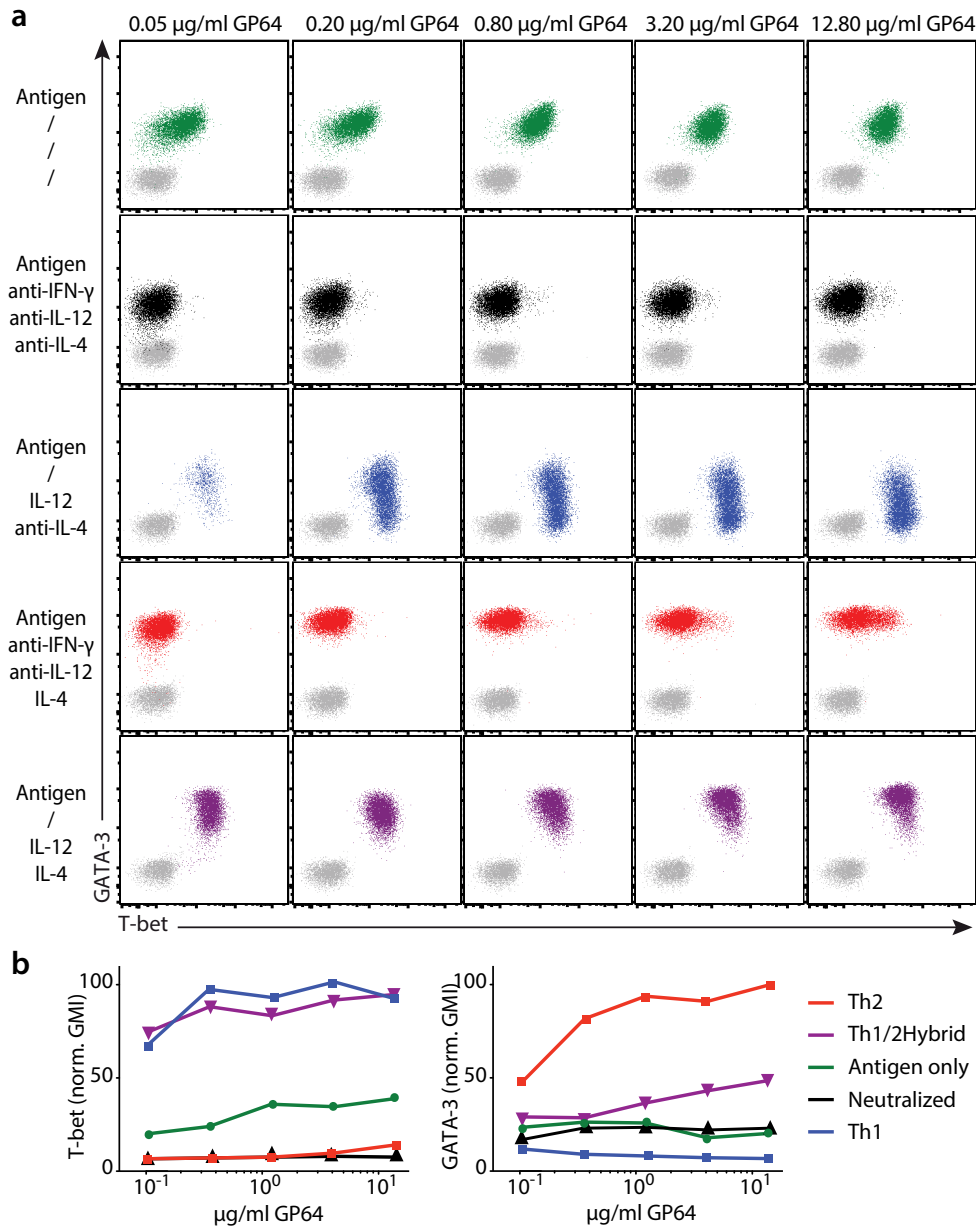
Figure 2.8: T-bet and GATA-3 are not differentially regulated by antigen. T-bet and GATA-3 flow cytometry measurements on day 4 of culture with increasing amounts of $GP64_{61-80}$ peptide without additional cytokines (green), with blockade of IFN-γ, IL-12 and IL-4 (neutralized, black), with IL-12 and anti-IL-4 (Th1, blue), with anti-IFN-γ, anti-IL12 and IL-4 (Th2, red) or IL-12 and IL-4 (hybrid Th1/2, purple). No IFN-γ was added, as the cells were IFN-γ competent and produced their own. **a** T-bet and GATA-3 stainings on day 4 of culture. **b** Dose responses of T-bet and GATA-3 to $GP64_{61-80}$ in the different conditions on day 4 of culture.
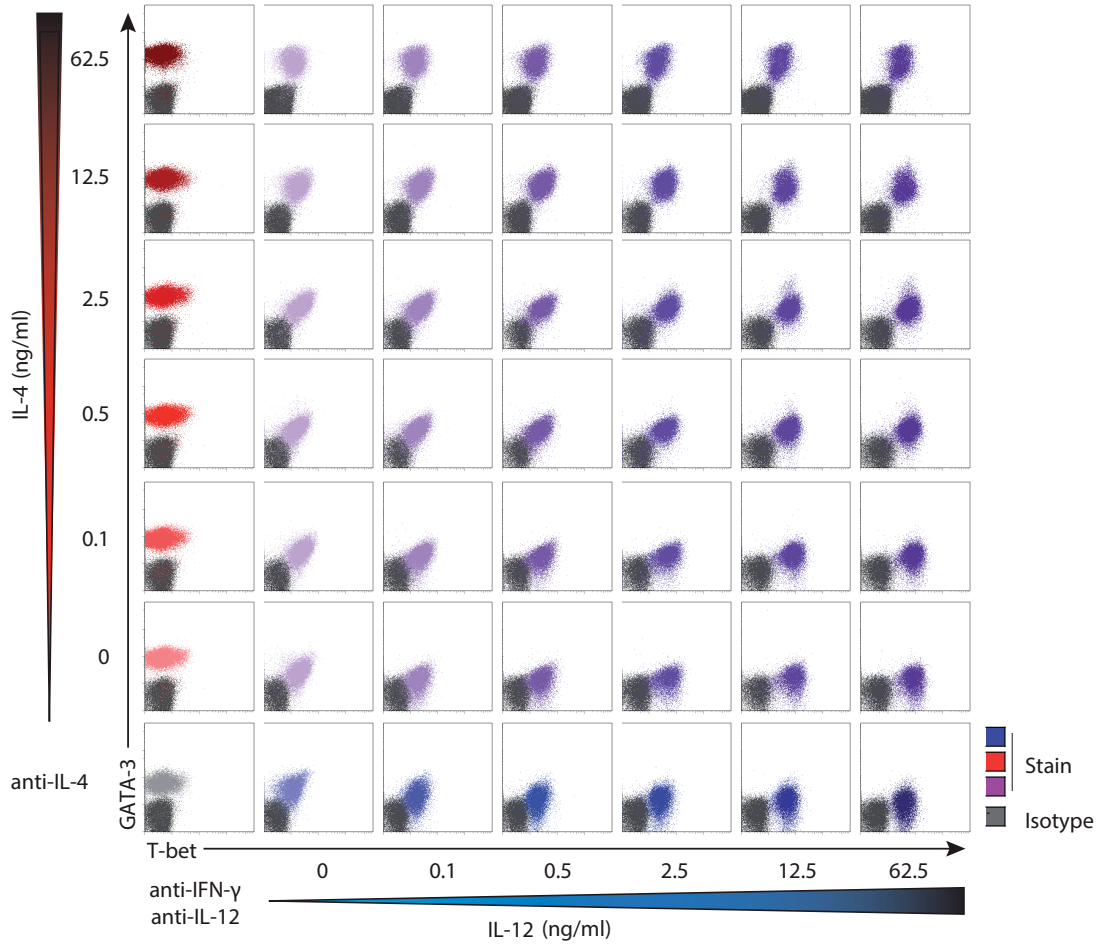
Figure 2.9: A continuum of T-bet and GATA-3 co-expressing states resulted from the titration of cytokine inputs. FACS-sorted naive IFN-γ- and IL-4-competent CD4 T cells were activated with APCs and GP$_{61-80}$ peptide under the indicated conditions. IL-12 and IL-4 were titrated against each other; no IFN-γ was added, as the cells were IFN-γ competent and produced their own. On day 5, T-bet and GATA-3 expressions were measured by flow cytometry.
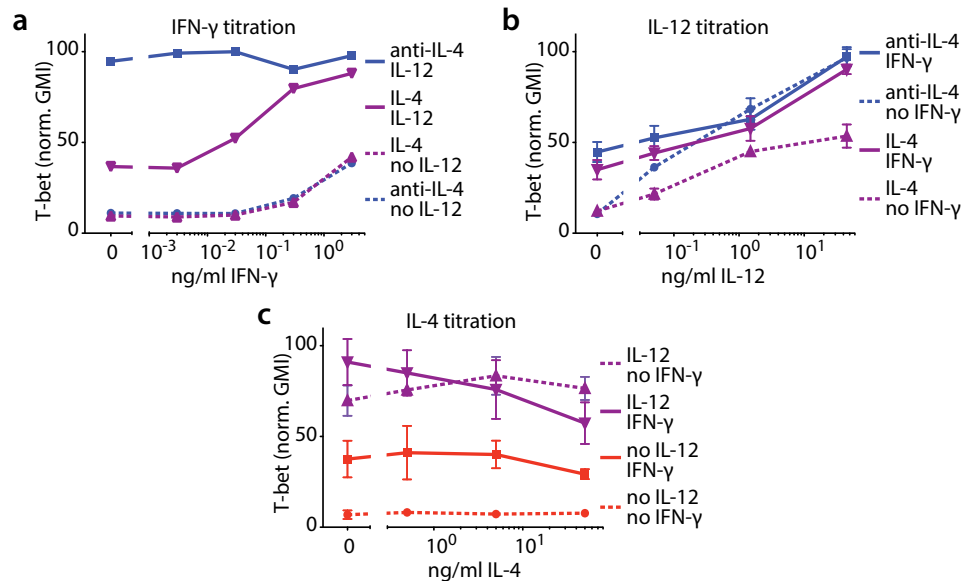
Figure 2.10: T-bet is strongly positively correlated to IFN-γ and IL-12. a Dose response of T-bet to IFN-γ with IL-12 (full lines) or without IL-12 (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **b** Dose response of T-bet to IL-12 with IFN-γ (full lines) or without IFN-γ (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **c** Dose response of T-bet to IL-4 with IFN-γ (full lines) or anti-IFN-γ (dotted lines), in the presence of IL-12 (purple lines) or without IL-12 (red lines).

absence of IL-12, the addition of IL-4 did not influence T-bet levels (compare the purple and blue dotted lines) and even high concentrations of IFN-γ were only associated to intermediate T-bet expression. Analysing the dose-response of T-bet to IL-12, we observed a similar dependence of T-bet to the IL-12 concentration than to the IFN-γ concentration: IL-12 correlated positively with T-bet amounts (Figure 2.10b). However, IL-12 was strictly needed to achieve optimal T-bet expression, both in the presence and absence of IFN-γ. The positive effect of IFN-γ could again be observed in hybrid conditions (compare full and dotted purple lines). The addition of IL-4 led to reduced T-bet expression and a decreased responsiveness to IL-12 in the absence of IFN-γ (compare blue and purple dotted lines), while IFN-γ in the absence on IL-4 had no effect on the dose-response of T-bet to IL-12. Finally, IL-4 showed no consistent effect on T-bet independently of IFN-γ and IL-12, but correlated negatively with the Th1 master transcription factors in the presence of IFN-γ (full lines). Collectively, these data show that cytokine effects on T-bet expression are strongly modulated by other cytokines.

We then analysed the response of GATA-3 to IL-4, IFN-γ and IL-12. First focusing on the titration of IL-4 (Figure 2.11c), its main regulator during Th2 differentiation, we observed a graded up-regulation of GATA-3 in response to increasing IL-4 concentrations. However, this effect seemed to be dampened in
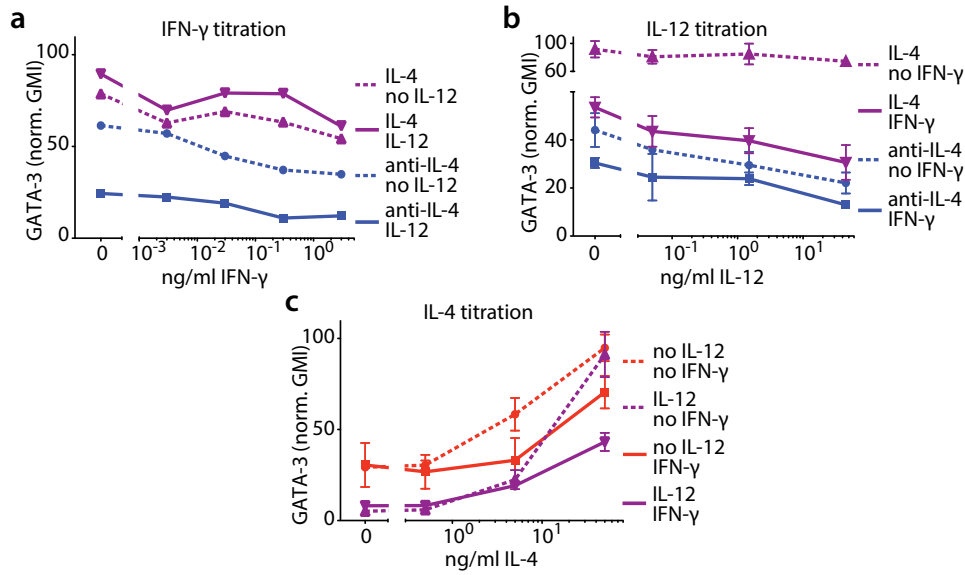
Figure 2.11: GATA-3 is strongly positively correlated to IL-4 and negatively to IFN-γ and IL-12. a
Dose response of GATA-3 to IFN-γ with IL-12 (full lines) or without IL-12 (dotted lines), in the presence of
IL-4 (purple lines) or anti-IL-4 (blue lines). **b** Dose response of GATA-3 to IL-12 with IFN-γ (full lines)
or without IFN-γ (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **c** Dose
response of GATA-3 to IL-4 with IFN-γ (full lines) or anti-IFN-γ (dotted lines), in the presence of IL-12
(purple lines) or without IL-12 (red lines).

the presence of IFN-γ; indeed, the up-regulation was stronger when IFN-γ was
added to the culture (compare full and dotted lines), while no reduction of GATA-3
expression could be observed in the absence of IL-4. Contrastingly, the addition of
IL-12 did not diminish the effect of IL-4, although it reduced the overall expression
of GATA-3 (compare red and purple lines). This negative effect of IL-12 seemed
to be overridden by high IL-4 concentrations (dotted purple line). Focusing on
the response of GATA-3 to IFN-γ (Figure 2.11a), we observed a dose-dependent
reduction of GATA-3 expression, most notably so in conditions where IL-4 was
absent (blue lines). In the presence of IL-4, the effect of IFN-γ was visible at low
concentrations and did not consistently increase with augmenting IFN-γ amounts
(purple lines). IL-12 did not influence the response to IFN-γ (compare full and
dotted lines). Finally, IL-12 also caused a gradual decrease in GATA-3 expression
(Figure 2.11b) in most conditions. In 'optimal' Th2 conditions (i.e., in the presence
of IL-4 and absence of IFN-γ, dotted purple line), however, IL-12 was not able
to suppress the IL-4-induced up-regulation of GATA-3; the presence of IFN-γ
restored the effect of IL-12 in the presence of IL-4 (full purple line), but had no
influence on the dose response in its absence. Thus GATA-3, like T-bet, is regulated
in a complex manner by multiple cytokine inputs.

The data on master transcitpion factor expression corroborated previously described mechanisms, like the up-regulation of T-bet by IFN-γ and IL-12 and the up-regulation of GATA-3 by IL-4. Distinct negative effects of both Th1 cytokines on GATA-3 could also be observed. Importantly, however, we found that the cytokines modulated each other's effects. The dose response of T-bet to IFN-γ was steeper in the presence of IL-12, and IL-4 prevented maximal T-bet up-regulation by IL-12 in the absence of IFN-γ. Conversely, the influence of IL-4 on GATA-3 was reduced in the presence of IL-12, and both IL-12 and IFN-γ were needed for maximal GATA-3 repression.

2.3.2   Total STATs expression is influenced by the concentration of polarising cytokines

The analysis of STAT phosphorylation showed negative effects of cytokines on STAT pathways, specifically of IL-4 on STAT4 phosphorylation at the end of differentiation. Therefore, we asked whether this negative cross-talk resulted from inhibition of STAT phosphorylation (i.e., mediated by SOCS proteins[179,180]) or from the regulation of STAT expression. Indeed, time-resolved flow cytometry quantification of total STAT1, STAT4 and STAT6 under Th1, Th2 and hybrid differentiation conditions showed that all three STAT proteins were dynamically and differentially regulated depending on the cytokine milieu (cf. Figure 2.4c): STAT1 and STAT4 were up-regulated in Th1 and hybrid Th1/2 cells whereas STAT6 expression was augmented in Th2 and hybrid Th1/2 cells. To determine how cytokines influenced STAT expression, we analysed the regulation of total STAT protein expression in response to IFN-γ, IL-12 and IL-4 by titrating each cytokine in the presence and absence of one or both of the others daily for five days. As in the analysis of T-bet and GATA-3 regulation, we discuss here the data for day 4, other days showing similar results.

The effect of IFN-γ, IL-12 and IL-4 on STAT1 expression are shown in Figure 2.12; the left panels depict typical stainings for all different cytokine combinations and the right panels the dose response to one cytokine in different conditions. Looking at STAT1 expression in response to IFN-γ (Figure 2.12a,b), we observed a gradual increase in protein levels with augmenting IFN-γ concentrations (Figure 2.12b). The positive correlation could be observed independently of the presence of IL-4 (compare blue and purple lines); however, the presence of IL-12 reduced the steepness of the dose-response (compare dotted and full lines). The positive effect of IFN-γ is also clearly visible in the histograms of Figure 2.12c (compare full and dotted lines). In contrast to IFN-γ, when analysing the dose-response of STAT1 to IL-12 (Figure 2.12c,d), we observed that the signal transducer was repressed by IL-12 (Figure 2.12d), although this down-regulation was only visible in the presence of IFN-γ (full lines). In the absence of IL-4 and IFN-γ, a slight positive correlation could be observed (dotted blue line). Finally, IL-4 showed no correlation with STAT1 expression (Figure 2.12e,f), but the titration confirmed the positive effect of IFN-γ (compare full and dotted lines in Figure 2.12f) and the negative effect of IL-12 in the presence of IFN-γ (compare full red and purple lines). Thus, STAT1 expression was up-regulated by IFN-γ and this activation was counteracted by IL-12.

The effect of IFN-γ, IL-12 and IL-4 on STAT4 expression are shown in Figure 2.13. The regulation of STAT4 by Th1 cytokines was qualitatively similar to that of STAT1. STAT4 expression was correlated positively to IFN-γ in all the

Figure 2.12: STAT1 correlates positively with IFN-γ and negatively with IL-12. a STAT1 expression in response to different cytokine combinations with and without IFN-γ. **b** Dose response of STAT1 to IFN-γ with IL-12 (full lines) or without IL-12 (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines).**c** STAT1 expression in response to different cytokine combinations with and without IL-12. **d** Dose response of STAT1 to IL-12 with IFN-γ (full lines) or without IFN-γ (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **e** STAT1 expression in response to different cytokine combinations with and without IL-4. **f** Dose response of STAT1 to IL-4 with IFN-γ (full lines) or anti-IFN-γ (dotted lines), in the presence of IL-12 (purple lines) or without IL-12 (red lines).

observed conditions (Figure 2.13a, b), although its overall levels were higher in the absence of IL-4 (Figure 2.13a, compare purple and blue lines). STAT4 correlated negatively to IL-12, despite the fact that IL-12 induces its phosphorylation (Figure 2.13c,d). This effect was augmented in the presence of IFN-γ (compare full and dotted lines in Figure 2.13c), but not modified by the addition of IL-4. Looking at the dose-response of STAT4 to IL-4, we observed that IL-4 induced a decrease in the STAT4 amount in the absence of the Th1-cytokine IFN-γ (Figure 2.13f, dotted lines), but had no effect otherwise. IL-12 did not influence the action of IL-4 on STAT4, but the overall decrease in STAT4 expression levels in its presence could be observed again (compare full and dotted lines). Collectively, these data show complex regulation of STAT4 protein levels by the cytokines, with an up-regulation downstream of IFN-γ and repression downstream of IL-12 and IL-4. Furthermore, IFN-γ augmented the decrease in STAT4 expression in response to IL-12.

Finally, we show the dose responses of the signal transducer STAT6 in figure 2.14. The dose response of IFN-γ showed a gradual negative effect of the latter on STAT6 (figure 2.14b), which was left unchanged by the addition of IL-12 or IL-4, even though the overall STAT6 levels were higher in the presence of IL-4 (compare purple and blue lines). IL-12 had no detectable effect on STAT6 expression (Figure 2.14c,d). The strongest response observed was to IL-4 (Figure 2.14e,f): STAT6 was strongly induced in response to increasing IL-4 concentrations (Figure 2.14f), suggesting a positive feedback-loop similar to that between STAT1 and IFN-γ. IFN-γ seemed to reduce IL-4-induced STAT6 up-regulation (compare full and dotted lines in figure 2.14f). Thus, STAT6 protein expression was positively regulated by IL-4 and negatively by IFN-γ.

> Interestingly, many different effects of the cytokines on STAT protein expression could be observed: while IFN-γ acted both to induce the Th1 factors STAT1 and STAT4 and to repress the Th2 factor STAT6, IL-12 acted as a Th1-brake on the protein expression level by repressing STAT1 and STAT4 expression while still supporting Th1 differentiation on the signalling level. The function of IL-4 was more restricted to the Th2 lineage in enhancing STAT6 expression.

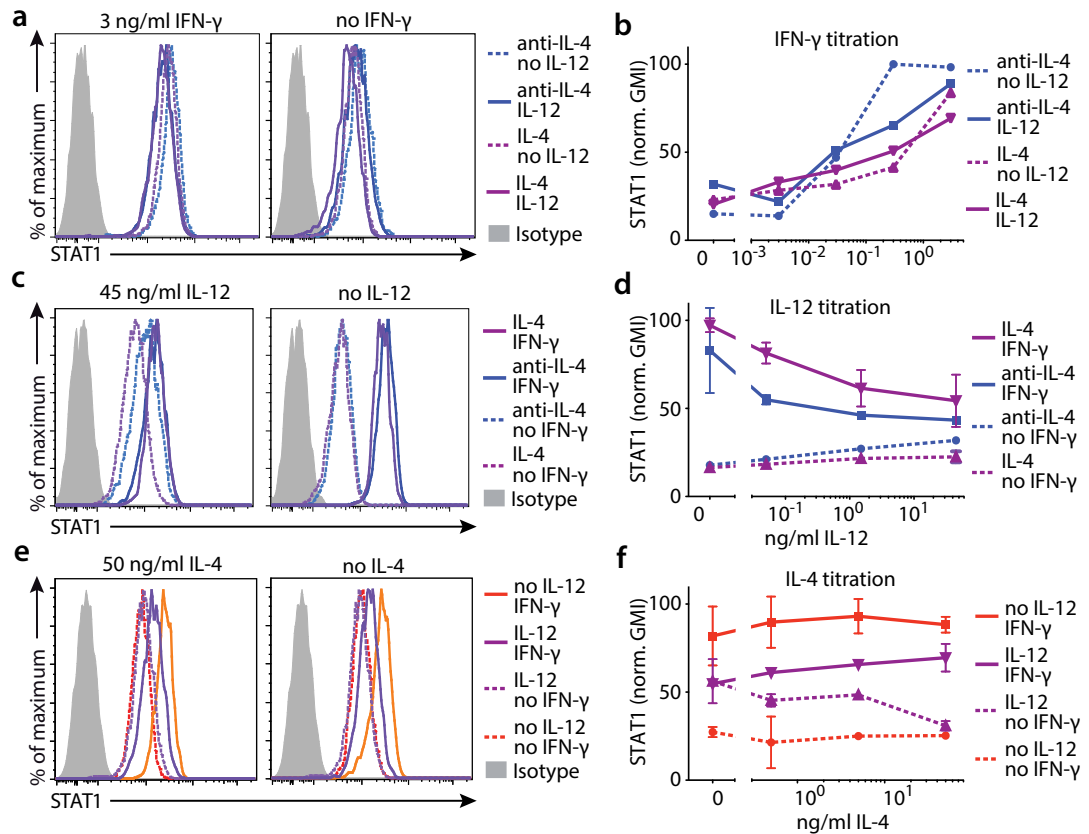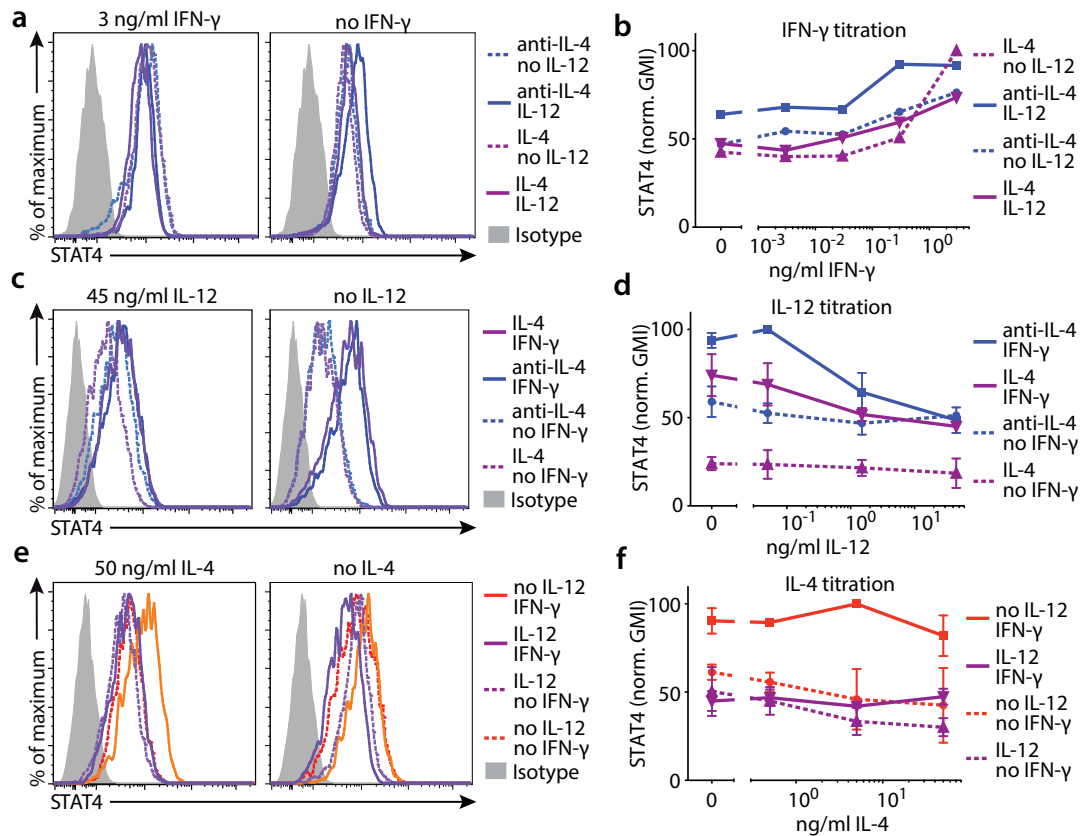**Figure 2.13: STAT4 correlates positively with IFN-γ and negatively with IL-12. a** STAT4 expression in response to different cytokine combinations with and without IFN-γ. **b** Dose response of STAT4 to IFN-γ with IL-12 (full lines) or without IL-12 (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **c** STAT4 expression in response to different cytokine combinations with and without IL-12. **d** Dose response of STAT4 to IL-12 with IFN-γ (full lines) or without IFN-γ (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **e** STAT4 expression in response to different cytokine combinations with and without IL-4. **f** Dose response of STAT4 to IL-4 with IFN-γ (full lines) or anti-IFN-γ (dotted lines), in the presence of IL-12 (purple lines) or without IL-12 (red lines).
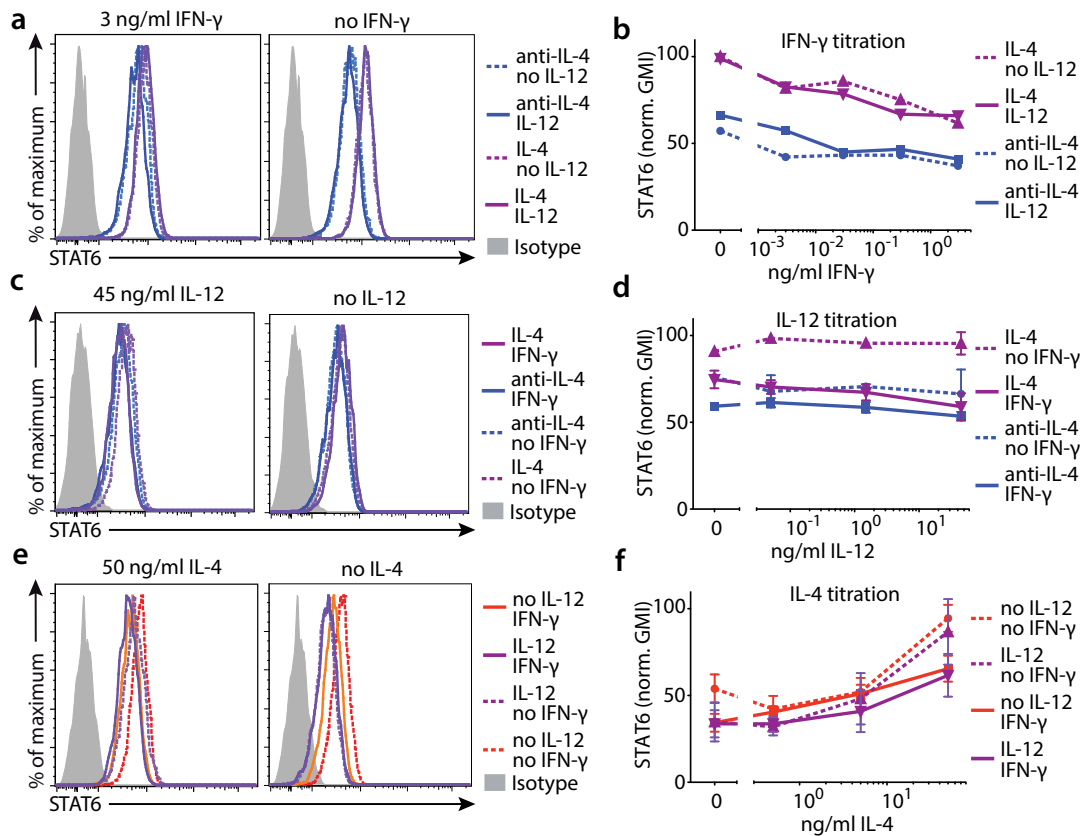
Figure 2.14: STAT6 correlates negatively with IFN-γ and positively with IL-4. a STAT6 expression in response to different cytokine combinations with and without IFN-γ. **b** Dose response of STAT6 to IFN-γ with IL-12 (full lines) or without IL-12 (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines).**c** STAT6 expression in response to different cytokine combinations with and without IL-12. **d** Dose response of STAT6 to IL-12 with IFN-γ (full lines) or without IFN-γ (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **e** STAT6 expression in response to different cytokine combinations with and without IL-4. **f** Dose response of STAT6 to IL-4 with IFN-γ (full lines) or anti-IFN-γ (dotted lines), in the presence of IL-12 (purple lines) or without IL-12 (red lines).

2.4 The amounts of polarising cytokines are predictive for the recall response of most cytokines

Th cells exert their function by secreting subset-specific effector cytokines both during differentiation in the course of a primary immune response and as memory cells, during a recall response triggered by a TCR stimulus (i.e., a secondary infection). TCR stimulation (here by addition of PMA and ionomycin, which mimick a strong TCR stimulus) of differentiated Th cells causes reactivation of the cytokine profile acquired during differentiation without the need for additional cytokine cues. Th1 cells secrete IFN-γ[46], whereas Th2 cells secrete IL-4, IL-10 and IL-13[47], both cell types secrete IL-2 and TNF-α, although they are preferentially expressed by Th1 cells[181]; hybrid subsets stably co-produce Th1 and Th2 cytokines[82,83,84]. IL-5 is also expressed by Th2[47] and hybrid cells; however, two weeks of consecutive differentiation are necessary to up-regulate IL-5 production *in vitro*, so that we do not consider it in this study. Binding of the master transcription factors to cytokine genes has been shown to result in epigenetic modifications[42]. To study the relation between MTF expression and cytokine recall response, we cultured IFN-γ- and IL-4-competent naive Th cells with a gradient of both IL-12 and IL-4 (without addition of IFN-γ, relying on the autogenous production by the cells) and measured T-bet and GATA-3 protein on day 5 (see figure 2.9), as well as IFN-γ, IL-4, IL-13, IL-10, TNF-α and IL-2 production after restimulation of the cells. The results are shown in the form of heat maps of T-bet and GATA-3 expression directly before the restimulation (also see Figure 2.9 for the flow cytometry plots and dose responses) and of cytokine production in terms of frequency of producing cells directly after restimulation in Figure 2.15a and b, respectively.

As observed previously, T-bet expression both increased with the IL-12 concentration (x-axis) and decreased with the IL-4 concentration (y-axis) (Figure 2.15a, upper panel), while the expression of GATA-3 increased with IL-4 and decreased with IL-12 (Figure 2.15a, lower panel). In addition to graded T-bet and GATA-3 co-expression patterns, we observed gradients in the frequency of cytokine producers, which were most pronounced for IFN-γ, IL-4, TNF-α and IL-2 (Figure 2.15b). The frequency of IFN-γ producers was increased by IL-12 and reduced by IL-4, showing a pattern similar to that of T-bet expression. While TNF-α and IL-2 also correlated positively with IL-12 and negatively with IL-4, the resulting expression patterns looked more like an inverse image of the GATA-3 pattern than like the T-bet pattern. Looking at Th2 cytokines, we observed inverse correlations between the frequency of IL-4 producers and the IL-12 and IL-4 concentrations added to the culture to those present for IFN-γ, TNF-α and IL-2: more added IL-4 led to more IL-4 producers, while more IL-12 reduced the number of IL-4 producers. Qualitatively, this resembled the GATA-3 expression heat map. IL-13 production,
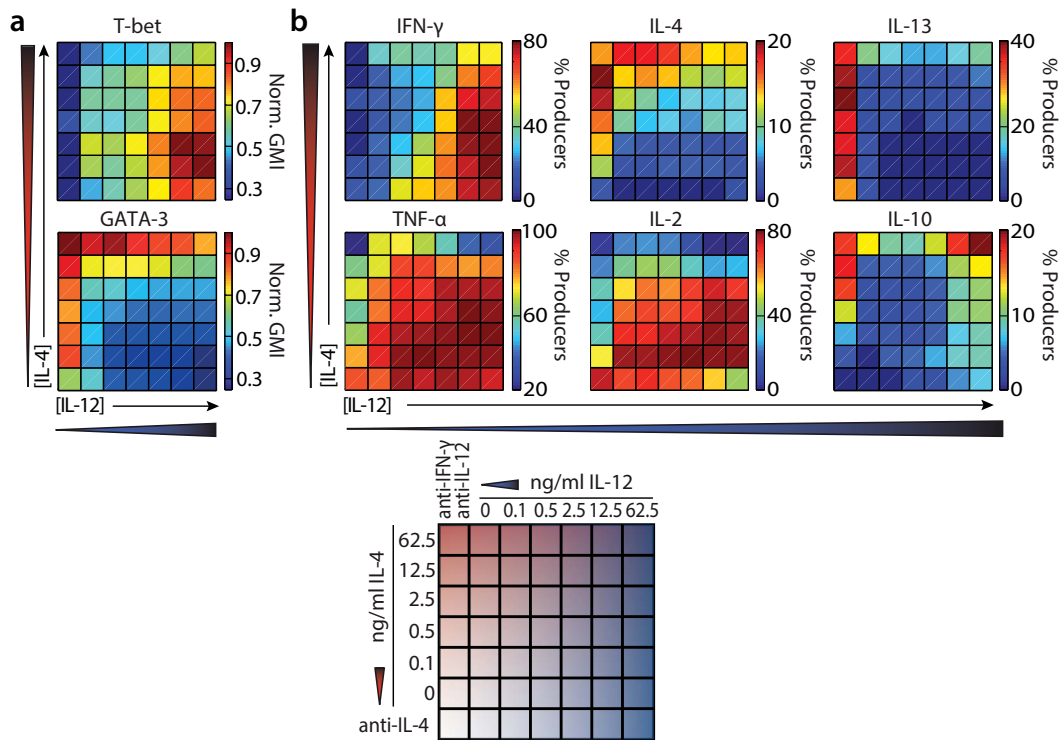
Figure 2.15: Cytokine expression largely reflects T-bet and GATA-3 expression. FACS-sorted naive IFN-γ- and IL-4-competent CD4 T cells were activated with APCs and GP_{61-80} peptide under the indicated conditions (legend on the right). On day 5, T-bet and GATA-3 expression levels were measured before the cells were restimulated with PMA and ionomycin in the presence of brefeldin A for 3 hours. Cytokines were then stained intracellularly. **a** Heatmap of T-bet and GATA-3 intensities before restimulation. **b** Heatmaps of IFN-γ, IL-4, IL-13, TNF-α, IL-2 and IL-10 percentages of producing cells.

although enhanced by the presence of added IL-4 (y-axis), seemed to be strongly repressed by Th1 polarising cytokines; indeed, the mere absence of anti-IFN-γ nearly abrogated its expression, even if high IL-4 concentration were used (second column). IL-10 production showed a complicated pattern: it was clearly positively correlated to the IL-4 concentration in the absence of Th1 cytokines (left column), but also enhanced by high IL-12 (right columns). Very little IL-10 could be detected with low to intermediate IL-12 concentrations in the absence of IFN-γ blockade, unless high IL-4 concentrations were used (centre part of the plot).

Several regulatory patterns seemed to exist for cytokine expression; IFN-γ, TNF-α and IL-2 correlated positively to IL-12 and negatively to IL-4, but while IFN-γ mirrored T-bet expression, TNF-α and IL-2 were inverse images of GATA-3 expression. IL-4 and IL-13 were increased by IL-4 and decreased by IL-12; IL-4 strongly mirrored GATA-3 expression, while IL-13 showed a mixed pattern. IL-10 production correlated to IL-4, but both this correlation and the frequency of producers were modulated by IL-12 in a complex manner.

2.5 Conclusion

Taken together, these results about STAT phosphorylation, master transcription factor expression, total STAT expression and cytokine production showed that graded stimuli induce graded responses and put multiple cross-effects between the Th1 and Th2 inducing pathways in evidence. The latter occurred at multiple levels: on the level of STAT phosphorylation, we observed phosphorylation of typically Th1 STATs downstream of IL-4; on the transcription factor expression levels, we observed repression of GATA-3 downstream of IFN-γ and IL-12 and of STAT6 downstream of IFN-γ; and finally, down-regulation of IL-13 production in response to Th1 cytokines. However, the piecemeal inspection of the multiple dose-response data did not yield a clear picture of the underlying regulatory interactions. The multiple correlations observed make it difficult to determine which factor influences which other factor, most of all if the whole dataset, and not only day four, is studied. We thus needed a more systematic approach to unravel the topology of the signalling network, which will be the topic of the next chapter.

Chapter 3

Linear regression analysis

In Chapter 2, we presented a series of titrations of the cytokines IFN-γ, IL-12 and IL-4 in differentiating T helper cells during which the downstream phosphorylation of STAT1, STAT4 and STAT6, respectively, as well as the total protein expression of the same STATs and T-bet and GATA-3 were measured. We analysed the dose responses of each of these nine protein species in order to gain insights into the integration of multiple signals resulting in Th1, Th2 and hybrid Th1/2 cells differentiation, as well as the cytokine production of the differentiated cells. Multiple correlations, both positive and negative, were put in evidence by a qualitative analysis of the data; however, the amount of data generated required a more systematic approach to clarify the relationships at play between the different transcription factors. Furthermore, as the protein amounts were measured using flow cytometry, a technology yielding quantitative results, a method providing quantitative information about the different mechanisms observed would allow a better analysis of the Th1/Th2 regulatory network studied.

Based on previous knowledge, we started by defining three layers of regulatees and regulators. The first layer is composed of the cytokines regulating STAT phosphorylation, the second of active transcription factors (i.e., the phosphorylated STATs, T-bet and GATA-3) regulating transcription factor expression, and the last of T-bet and GATA-3 regulating cytokine production. Although activated STATs have been shown to be involved in cytokine regulation, the protocol used in this study focused on cytokine expression in differentiated cells following TCR stimulation in the absence of polarising cytokines, and thus in the absence of relevant amounts of phosphorylated STATs. We therefore omitted the latter in our analysis of cytokine expression. To systematically analyse the titration data presented in Chapter 2 in order to derive the topology of the regulation network, we used a series of linear regression models describing how a layer of regulators
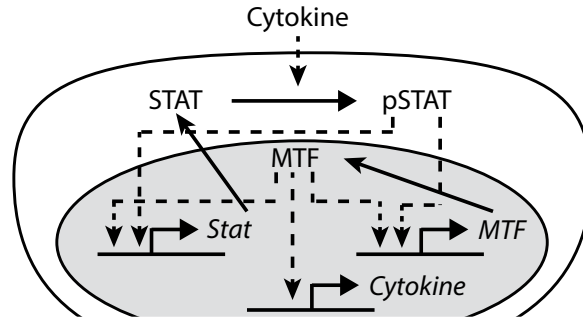
Figure 3.1: Mechanistic assumption for Th cell differentiation. We assumed that the cytokines mediate STAT phosphorylation, allowing the active pSTATs to regulate the expression of the master transcription factors and the STATs. The master transcription factors in turn regulate each other's expression, the expression of the STATs and cytokine production upon restimulation.

controls a corresponding layer of regulatees:

$$\text{regulatee}_i = \alpha_0 + \sum_j \alpha_j \cdot \text{regulator}_j$$

The advantages of linear regression models are that they allow a straightforward and unbiased analysis of trends in the data with very few assumptions. In our cases, the only assumption was the hierarchy of the network presented in Figure 3.1, which is based on reliable biological knowledge: cytokines induce STAT phosphorylation and transcription factors regulate the transcription of genes.

To allow for rigorous model selection, we considered a family of models composed of all possible combinations of the potential regulators of each regulatee. Specifically, this family contains all models with single regulators, all regulator pairs, triplets, and so on. The models describing the amounts of phosphorylated STATs, total STATs, T-bet, GATA-3 and fraction of cytokine producers were fitted to the normalised data derived from all the titrations performed in order to determine the values of the regression coefficients α_j. We tested for each regulator wether it made a significant contribution to the levels of the regulatee using the F-statistic in analysis of variance (ANOVA) calculations[182]. In order to compare the models for each regulatee and determine which of the regulators were significant for its regulation, we ranked the models by goodness of fit (measured by the coefficient of determination, R^2) and Akaike information criterion[183,184](AIC). R^2 ranges from 0 to 1 and indicates the fraction of the data that is explained by the model while the AIC additionally implements a trade-off between complexity of the model and goodness of fit, allowing to quantify the quality of each model relative to the others. The model with the lowest AIC, the highest R^2 and no non-significant parameter

was the most likely to explain the experimental data. We further inspected the quality of the model fits using (i) Cook's distance, which put outliers in evidence[182], and (ii) the probability plots of the residuals as well as the residuals themselves to ascertain their normal distribution and the absence of bias in the model[182]. This analysis and the associated statistics were computed using Wolfram Mathematica 10 (see Section 6.2.2 for more details).

3.1   STAT activation is induced, but not repressed, by both canonical and non-canonical cytokines

The first layer of regulation downstream of the polarising cytokines is the phosphorylation of the STATs. The visual analysis of the data presented in the previous chapter suggested the presence of several cross-effects between cytokines on STAT phosphorylation. Such positive effects could be due to activation of non-canonical STATs by cytokines or to up-regulation of signalling components, whereas negative effects could be caused by the induction of negative regulators of STAT phosphorylation (e.g., SOCS proteins[179,180]) or down-regulation of components of the signalling pathway. We analysed the effect of the cytokines on the phosphorylation level systematically. As dynamic regulation of the total STAT expression is also reflected in the amount of pSTAT that is measured, without it being a direct effect on phosphorylation, we computed the relative pSTAT values which inform us about the fraction of protein being phosphorylated independently of the total amount expressed. The idea is that STAT phosphorylation is a fast process (typically happening in 10 to 20 minutes) while changes in STAT expression occur on much larger timescales. The relative pSTAT values were obtained by co-staining pSTATs and total STATs during the experiments and dividing the pSTAT values by the corresponding total STAT values after normalisation:

$$\text{pSTAT}_{\text{rel},i} = \frac{\text{pSTAT}_i - \text{baseline}}{\text{STAT}_i}$$

for $i = 1, 4, 6$ and each experiment. The baseline was set to be equal to the lowest normalised pSTAT value for each experiment and each day.

We began by considering all possible models of how three cytokine stimuli could control the phosphorylation of each of the three STATs, resulting in seven models for each pSTAT (Figure 3.2). As input variables, we chose the logarithms of the cytokine concentration,
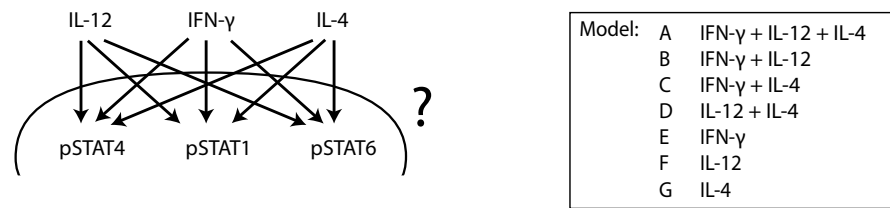
Figure 3.2: Possible ways of STAT phosphorylation. The general assumption that each cytokine can induce or repress the phosphorylation of each STAT is tested by comparing models representing all the different cytokine combinations.

motivated by the fact that receptor occupancy saturates. We performed this linear regression analysis separately for each day.

$$pSTAT_{rel\ i} = \beta_{1\ i}\log(IFN\text{-}\gamma) + \beta_{2\ i}\log(IL\text{-}12) + \beta_{3\ i}\log(IL\text{-}4)$$

for $i = 1\ 4\ 6$ and allowing each $\beta_{j\ i}$ to be equal to zero; i.e., each cytokine to have no effect at all on the phosphorylation of each STAT. The fitting to the relative pSTAT data (ranged between 0 and 1) allowed us to compare the regression coefficients obtained for different days.

The ranking of the models by goodness of fit and AIC is shown in Figure 3.3 for day 1 for pSTAT1 and day 3 for pSTAT4 and pSTAT6 (the days shown here were those with the best-fitting models, see Table 3.1 for the other time points). We selected the best model for each pSTAT according to the value of the AIC: the model with the lowest AIC was best able to fit the data with only significant explanatory variables. Standard diagnostics such as the plotting of Cook's Distance, computing the probability plots of the residuals and residual inspection were performed to confirm that the model fits were satisfactory (Supplementary Figure 7.4).

Figure 3.3: STAT phosphorylation can be induced by several non-canonical cytokines. The best model is boxed and is the one with the lowest AIC, the greatest R^2 and only significant regressors. Left: models describing pSTAT1 on day 1; middle: models describing pSTAT4 on day 3; right: models describing pSTAT6 on day 3.

Using these linear modelling approach, we can indeed explain much of the measured variability in the pSTAT data on most days with the combined effects of the cytokine stimuli (Table 3.1; coefficients of determination, R^2, for pSTAT1 0.32 to 0.75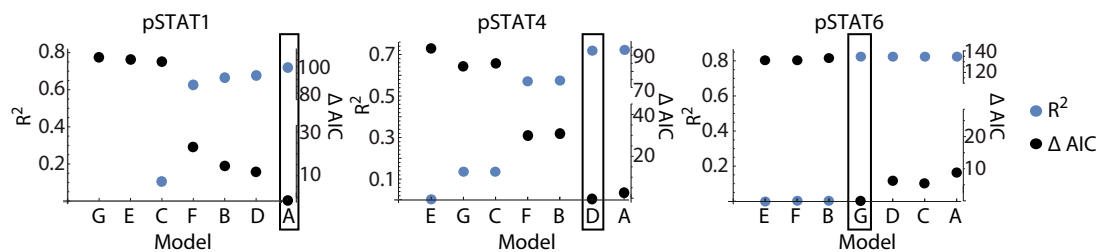, for pSTAT4 0.62 to 0.75 and for pSTAT6 0.47 to 0.88). The finding that all regression parameters are non-negative implies that cytokines only activate (and never inhibit, in this particular setup) STAT phosphorylation (Table 3.1). This finding implies that potential inhibitory cytokine effects are not due to crosstalk in signal transduction.

The regression analysis identifies the known canonical signalling pathways (i.e., pSTAT1 activation by IFN-γ, pSTAT4 activation by IL-12 and pSTAT6 activation by IL-4). In addition, we find weaker, non-canonical activations: phosphorylation of STAT1 by IL-12, as well as phosphorylation of STAT1 and STAT4 by IL-4. Looking at the kinetics of STAT1 phosphorylation, we observe a decrease in goodness of fit from day 3 on accompanied by a reduction in the strength of the IFN-γ effect, which corresponds to the time point where pSTAT1 amounts decreased. In contrast, pSTAT4 levels are well explained during the whole time course; however, the IL-12 effect becomes stronger with time, reflecting the late increase in pSTAT4. pSTAT6 phosphorylation is only linked to IL-4, and this effect remains stable with time. We summarised these results by averaging the regression coefficients over days 1 to 5 for each interaction (Figure 3.4a, hitherto undescribed effects are shown as green arrows here and later on).

The predictions of the regression modelling were verified directly by flow cytometry; plots of the response of the pSTATs to their non-canonical cytokines on day 3 of culture are shown in Figure 3.4b. The left panel shows the phosphorylation of STAT1 (top) and the absence of phosphorylation of STAT6 (bottom) in response to IL-12; STAT1 phosphorylation was increased by 45 ng/ml IL-12 2.5 times above baseline. The middle panel shows that neither STAT4 nor STAT6 phosphorylation were increased by IFN-γ. Finally, the right panel shows a 2-fold increase of STAT1 phosphorylation and a 2.9-fold increase of STAT4 phosphorylation in the presence of IL-4 compared to the blockade of IL-4.

		pSTAT1				pSTAT4			pSTAT6	
Day	IFN-γ α_1	IL-12 α_2	IL-4 α_3	R^2	IL-12 α_2	IL-4 α_3	R^2	IL-4 α_3	R^2
1	0.67	0.21	0.26	0.75	0.34	0.28	0.63	0.91	0.88
2	0.6	0.4	0.7	0.7	0.63	0.17	0.7	0.88	0.85
3	0.15	0.46	0.39	0.39	0.63	0.27	0.75	0.85	0.85
4	0.2	0.26	0.34	0.34	0.69	0.15	0.69	0.91	0.81
5	0.19	0.1	0.32	0.32	0.7	0.17	0.62	0.73	0.47
Mean	0.36	0.29	0.2	0.5	0.6	0.21	0.68	0.86	0.78

Table 3.1: Best-fit parameters and R^2 for pSTAT1, pSTAT4 and pSTAT6 on each day
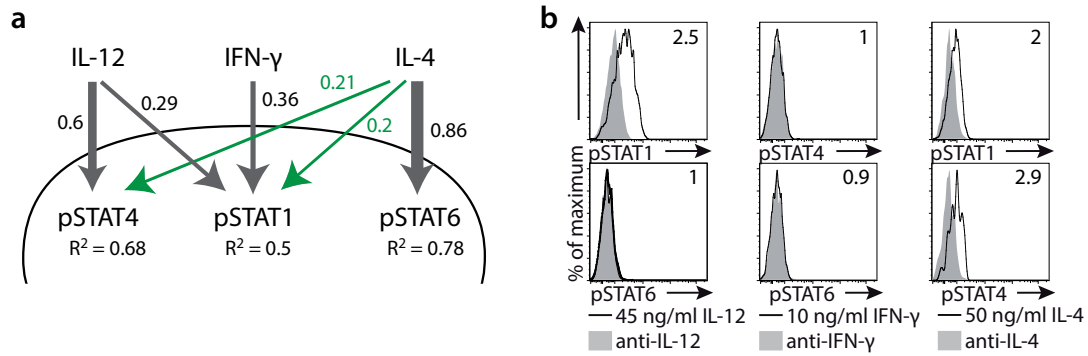
Figure 3.4: Observed ways of STAT phosphorylation. a Graphical representation of the best linear models describing the effect of IL-12, IFN-γ and IL-4 on STAT1, STAT4 and STAT6 phosphorylation. Grey arrows represent previously described interactions, green arrows hitherto undescribed mechanisms. Numbers show the average strength of the effect the cytokines have during the 5 days of culture. **b** Flow cytometry measurements of the non-canonical pSTATs downstream of IL-12 (left), IFN-γ (middle) and IL-4 (right) on day 3 of culture.

> Linear regression analysis allowed us to quantify statistically significant effects of polarising cytokines on STAT phosphorylation. The challenge of naive T cells with mixed Th1-Th2 cytokine stimuli revealed canonical and non-canonical cytokine-induced STAT phosphorylation. Specifically, in addition to the well-studied and dominating activation of STAT1 by IFN-γ, STAT4 by IL-12 and STAT6 by IL-4, the activation of STAT1 and STAT4 by IL-4 as well as the activation of STAT1 by IL-12 was observed. No negative regulation of STAT phosphorylation by IFN-γ, IL-12 or IL-4 was observed.

3.2 Transcription factor expression can be explained by linear combinations of the weighted sums over time of active transcription factors

Having established how the pSTATs depended on the cytokine inputs, we moved on to the next layer of the Th1/Th2 regulatory network and analysed how transcription factor expression is regulated. Complex interdependencies of the cytokines in influencing the transcription factor expression observed in the data were described in the previous chapter, which we again sought to capture by linear regression models. Because cytokine signalling is carried out by pSTATs and the master transcription factors and STATs are known to be their direct targets, and because previous work has indicated that MTFs can affect each other's expression, we used the pSTATs and T-bet as explanatory variables (regulators) for GATA-3 expression, the pSTATs and GATA-3 for T-bet expression, and the pSTATs, T-bet and GATA-3 for STAT1, STAT4 and STAT6 expression (Figure 3.5). Although GATA-3 and T-bet are known to enhance their own expression though several mechanisms, the master transcription factors could not be included in the linear models describing their own expression; indeed, auto-activating loops cannot be represented in linear models, as they only quantify the correlation between factors and one factor always correlates 100% with itself (so that T-bet would be best explained by only T-bet itself, for example). However, we expect that this is not a major restriction of the analysis, because auto-activation has primarily been implicated in the maintenance of master transcription factor expression, whereas we study the induction phase. In contrast to the master transcription factors, we had separate measurements for the total and active, phosphorylated STATs, allowing us to use, for example, pSTAT1 as a regulator for total STAT1. The distinct measurements of pSTATs, used as regulators, and total STATs, which are regulatees, allowed the identification of positive feedback loops of the phosphorylated STATs on the total STATs. No such distinction could be made for the master transcription factors, and a variable cannot be simultaneously regulator and regulatee in linear models. Hence models could not include auto-activation of T-bet and GATA-3.

3.2.1 The regulation of STAT expression happens on a slower timescale than the regulation of T-bet and GATA-3

In contrast to the regulation of STAT phosphorylation by the cytokines, which is a fast process, regulation of expression can affect the protein levels for several days thanks to a longer protein half-live and the effect of epigenetic modifications, so that we considered the possibility that the regulatory effects of the transcription factors were cumulative over time. Indeed, when measuring the amount of a
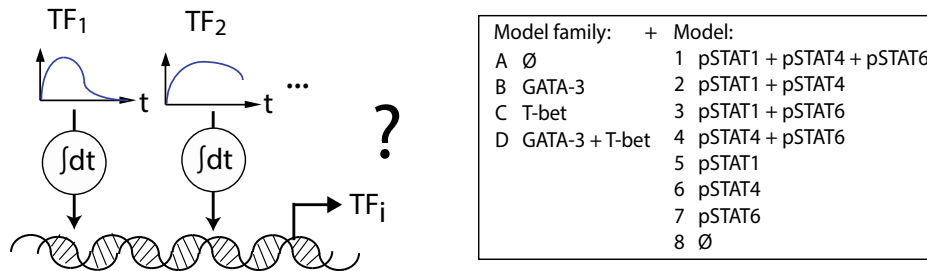
Figure 3.5: Possible ways of transcription factor transcription regulation. The general assumption is that each transcription factor can contribute in a negative or positive manner to the expression of another transcription factor, and that these contributions are cumulative over time, leading to up to 32 different models for a transcription factor. Possible models for T-bet are A+i and B+i ($i = 1...8$), for GATA-3, A+i and C+i ($i = 1...8$), and for all three STATs X+i ($X = A...D$ and $i = 1...8$).

protein species at a certain time point, not only the acutely induced protein can be detected, but also the proteins produced at earlier time points still present in the cell. Furthermore, we postulated that the effect of signalling on the transcription factor expression decreased with time, e.g., that signalling occurring on day 1 had less influence on the expression on day 4 than signalling occurring on day 4 due to protein degradation, and that the correlation between a regulatee and a regulator would therefore be stronger when quantified at the same time point than if the regulator was measured at an earlier time. We thus express the relationship between a regulatee and its regulators as follow:

$$\text{TF}_{i,T} = \sum_j \alpha_j \cdot \sum_t^T e^{-\lambda \cdot (T-t)} \cdot \text{TF}_{j,t}$$

for $T = 1...5$ and all combinations of j for each i, and allowing each $\alpha_j = 0$; i.e., all possible combinations of transcription factors. The factor $e^{-\lambda \cdot (T-t)}$ is the weight of the regulators depending on the time point t of their measurement. We thus had 16 different models for T-bet and GATA-3, and 32 for each of the STAT proteins (Figure 3.5).

When visually analysing the data, we chose to focus our analysis on day 4, as the Th cells had already reached a distinct phenotype by that point as judged by master transcription factor expression. Hence we used the weighted sum of the pSTATs as well as of T-bet and GATA-3 amounts from day 1 to 4 to explain the total expression of the transcription factors on day 4, but still considered the similar analysis performed on the other days which yielded similar results. Indeed, the regression analysis was performed in the same fashion using the weighted sum from day 1 to day n to explain the expression on day n, where $n = 1, 2, 3, 4, 5$; the
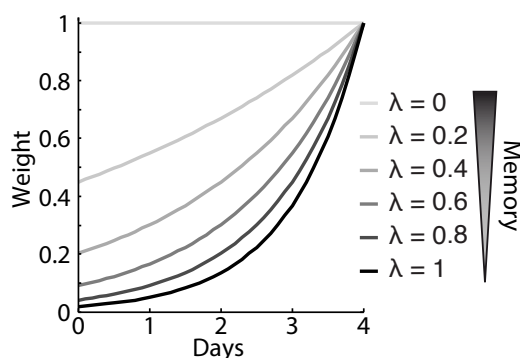
Figure 3.6: Influence of λ on the weight each day. The functions $e^{-\lambda \cdot (T-t)}$ with $T = 4$ and $t = 1...4$ describe the decrease of the weight of each transcription factor with time for different values of λ.

		T-bet	GATA-3	STAT1	STAT4	STAT6
λ (days^{-1})		0.8	1	0.4	0.2	0.4

Table 3.2: λ values of the best fits

qualitative and quantitative results (i.e., which regressors were significant as well as their weight) of the regression analysis for the different days were compared and found to be consistent over time. We started by estimating the best value for λ, which represents the 'memory' of the regulated transcription factor for previous influence of other transcription factors. The influence of the value of λ on the weight given to each day is illustrated in Figure 3.6: the higher λ is, the less early time points influence the expression at later time points; e.g., with $\lambda = 0$, all time points have the same weight, while with $\lambda = 1$, the weight of day 1 is less than one tenth of that of day 4 in explaining the expression of the regulatee on day 4. To estimate λ, we used the most complex linear model of each family (always leading to the best R^2), fitted the α coefficients for increasing λ values and compared the resulting R^2. The value of λ leading to the highest R^2 was used for further analysis. We obtained the best fits to the data for all transcription factors if earlier time points retained an influence on controlling the final expression level but with reduced weight (Figure 3.7 and Table 3.2). Specifically, the earlier time points seem to be more important for the STAT proteins (with λ values of 0.4, 0.2 and 0.4 for STAT1, STAT4 and STAT6, respectively) than for the master transcription factors (with λ values of 0.8 and 1 for T-bet and GATA-3, respectively).

> The expression of the transcription factors show a 'memory' for previous transcription factor activity as it is influenced by the expression of regulators several days earlier. This early expression of the regulators has a more important effect on the STATs than on the master transcription factors.
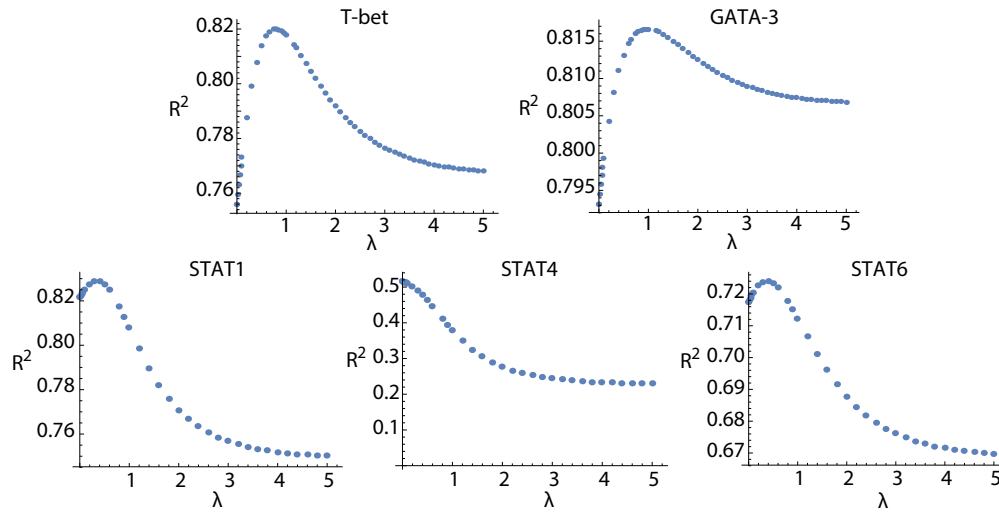
Figure 3.7: Regulation on earlier days have less influence on transcription factor expression than regulation directly preceding the measurement. The most complex model for each transcription factor was fitted with different values for λ for day 4 of culture and the resulting R^2 were compared.

3.2.2 T-bet expression is inhibited by GATA-3, while GATA-3 expression is repressed by pSTAT1 and pSTAT4

Master transcription factors are crucial for determining the phenotype of Th cells after differentiation. In Chapter 2, we observed the expected up-regulation of T-bet downstream of IFN-γ and IL-12 and of GATA-3 downstream of IL-4, but also inter-lineage correlations as GATA-3 expression was repressed by both Th1 cytokines. However, as several transcription factors correlate positively with one cytokine (e.g., pSTAT4, pSTAT1 and T-bet with IL-12), classical analysis of the data could not tell on which transcription factors were responsible for the observed effects. In order to study and quantify these interactions and systematically search for other possible regulatory mechanisms, we proceeded to analyse the regulation of T-bet and GATA-3 during our titration experiments using a linear regression analysis approach. We used the weighted sums over time of the pSTATs and T-bet or GATA-3 as explanatory variables for GATA-3 or T-bet, respectively, as well as the values of λ determined as described in the previous section for the weighting factors (c.f. Table 3.2 and Figures 3.5, 3.6 and 3.7). We then fitted all possible models to the titration data and compared the results for the different models in each family in order to find the best-fitting one. To do so, we classified the models by R^2 and AIC values, and selected the model with the lowest AIC comprising only parameters that were significantly different from zero (Figure 3.8). As for the pSTAT models, we ascertained that the standard model diagnostics of the selected models were satisfactory (Supplementary Figure 7.5).
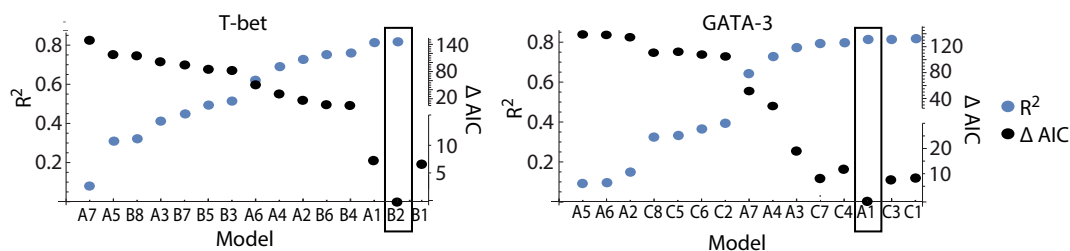
Figure 3.8: T-bet is regulated by pSTAT1, pSTAT4 and GATA-3, while GATA-3 is only regulated by the pSTATs. The best model is boxed and is the one with the lowest AIC, the greatest R^2 and only significant regressors. Left: models describing T-bet on day 4; right: models describing GATA-3 on day 4.

	T-bet				GATA-3			
Day	pSTAT1	pSTAT4	GATA-3	R^2	pSTAT1	pSTAT4	pSTAT6	R^2
1	0.98	0	0	0.8	-0.31	-0.03	0.89	0.74
2	0.92	0.18	-0.1	0.87	-0.17	-0.13	0.87	0.78
3	0.58	0.47	-0.24	0.83	-0.3	-0.21	0.87	0.84
4	0.34	0.62	-0.32	0.82	-0.35	-0.21	0.82	0.81
5	0.18	0.69	-0.34	0.81	-0.36	-0.35	0.68	0.59

Table 3.3: Best-fit parameters and R^2 for T-bet and GATA-3 on each day.

The values corresponding to the best fit for each model family are shown in Table 3.3 and represented graphically for day 4 in Figure 3.9a. The regression models fit the data very well ($R^2 = 0.82$ and 0.81 for T-bet and GATA-3, respectively, on day 4). They recover the known mechanisms of T-bet up-regulation by pSTAT4 and pSTAT1, and of GATA-3 up-regulation by pSTAT6. Surprisingly, no direct role for T-bet in GATA-3 regulation emerges: the negative regulation of GATA-3 observed downstream of IFN- and IL-12 is predicted to be carried out by both pSTAT1 and pSTAT4. Moreover, T-bet is predicted to be inhibited by GATA-3 and not by pSTAT6. In this respect it is interesting that no clear negative correlation to IL-4 could be observed in our previous qualitative analysis, supporting an effect mediated by GATA-3 which is regulated by more than IL-4 rather than a direct effect of pSTAT6 which is tightly correlated to the IL-4 concentration.

The kinetic nature of the data allowed us to do a linear regression analysis for the expression on each day in order to study the changes in regulation during the week of differentiation. The best models were able to explain T-bet and GATA-3 expression well on all five days (R^2 for T-bet 0.8 to 0.87 and for GATA-3 0.59 to 0.84, Table 3.3). The strength of regulation of pSTAT1 and pSTAT6 on GATA-3 show little consistent change with time, while the effect of pSTAT4 increases during differentiation. However, when considering T-bet regulation on the different days (Table 3.3 and Figure 3.9b), a decrease in the strength of regulation by pSTAT1 can be observed from day 2 on, while the effect of both pSTAT4 and GATA-3 increase until the end of the kinetics.
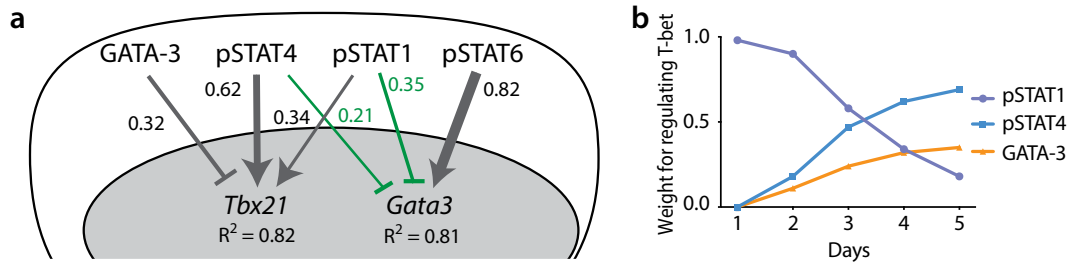
Figure 3.9: Observed ways of MTF regulation. a Graphical representation of the best linear models describing the effect of pSTAT1, pSTAT4, pSTAT6 and GATA-3 on T-bet and GATA-3 expression. Grey arrows represent previously described interactions, green arrows hitherto undescribed mechanisms. Numbers show the strength of regulation on day 4. **b** Evolution of the relative weights (absolute values) of pSTAT1, pSTAT4 and GATA-3 regulation on T-bet during the culture.

Linear regression analysis predicts T-bet to be up-regulated by pSTAT1 and pSTAT4, the first having a greater effect at earlier time points and the latter at late time points. GATA-3 is predicted to have an increasing negative effect on T-bet. On the Th2 side, GATA-3 is predicted to be positively regulated by pSTAT6 with little change over time, and negatively regulated by both pSTAT1 and pSTAT4, the latter having an increasing effect with time.
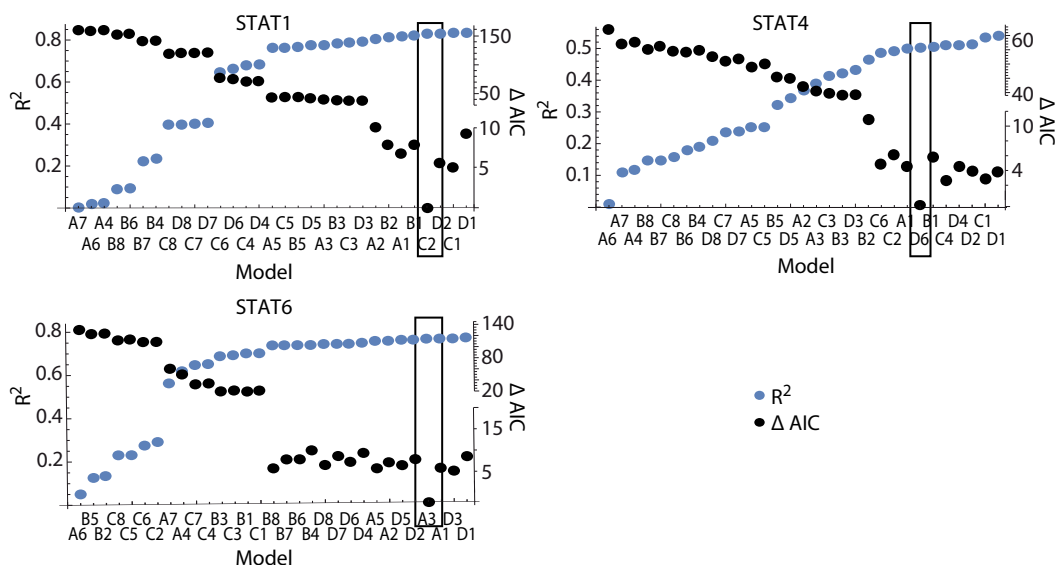
Figure 3.10: pSTATs have an important role in the regulation of STAT expression. The best model is boxed and is the one with the lowest AIC, the greatest R^2 and only significant regressors. Top left: models describing STAT1 on day 4; top right: models describing STAT4 on day 4.; bottom; models describing STAT6 on day 4.

3.2.3 Total STAT expression is strongly regulated by the pSTATs and master transcription factors

In Chapter 2, we observed dynamical regulation of the total protein expression of STAT1, STAT4 and STAT6 downstream of IFN- , IL-12 and IL-4. STAT1 and STAT4 were positively correlated to IFN- and negatively to IL-12, while STAT6 was strongly induced in the presence of IL-4 and repressed by IFN- . Furthermore, STAT4 expression decreased in response to IL-4 in certain conditions. As was the case for T-bet and GATA-3, simple visual analysis of the data did not allow for the quantification of the observed regulation, nor for the determination of the transcription factor responsible for the observed effect. We thus proceeded to systematically extract the regulatory network underlying total STAT expression regulation, using the same regression analysis described above: as the most prominent cytokine-specific active transcription factors are the pSTATs and T-bet and GATA-3, we took them as explanatory variables for the regression models, analogous to the models for T-bet and GATA-3 expression above, and included a weighted sum of the regulators with the previously determined values for (c.f. Table 3.2 and Figures 3.5, 3.6 and 3.7). After fitting all possible models to the titration data, we selected the best models according to the R^2 and AIC values (Figure 3.10) and verified standard model diagnostics (Supplementary Figure 7.6).

	STAT1				STAT4			
Day	pSTAT1	pSTAT4	T-bet	R^2	pSTAT4	T-bet	GATA-3	R^2
1	1.1	-0.16	0	0.79	0	0	0	0
2	0.9	-0.47	0.4	0.86	0	0.35	-0.16	0.21
3	0.92	-0.6	0.38	0.8	-0.18	0.46	-0.29	0.3
4	0.88	-0.44	0.39	0.83	-0.71	0.81	-0.12	0.51
5	0.92	-0.41	0.22	0.87	-0.41	0.78	-0.18	0.43

	STAT6		
Day	pSTAT1	pSTAT6	R^2
1	0	0.73	0.52
2	0	0.76	0.57
3	-0.35	0.79	0.67
4	-0.49	0.78	0.72
5	-0.39	0.56	0.38

Table 3.4: Best-fit parameters and R^2 for STAT1, STAT4 and STAT6 on each day.

We again achieved good fits to the collective data on day 4 (R^2 = 0.83, 0.51 and 0.72 for STAT1, STAT4 and STAT6, respectively; Table 3.4 and Figure 3.11). The models reveal four positive and four negative regulations of STAT expression on day 4 (Figure 3.11). All positive interactions act within the Th1 or Th2 pathways. By contrast, negative regulations are observed between the Th1 and Th2 pathways, but also within the Th1 pathway. STAT1 is found to be up-regulated by both pSTAT1 and T-bet, but repressed by the Th1 factor pSTAT4. STAT4 is regulated in a similar fashion: its expression is increased by T-bet and repressed by pSTAT4; in addition, it is down-regulated by GATA-3. STAT6 is induced by pSTAT6 and down-regulated by pSTAT1.

Kinetic analysis show that the effect of pSTAT1 on STAT1 expression stays relatively constant, while those of pSTAT4 and T-bet on STAT1 tend to increase with time. This can also be observed when considering STAT4 regulation; the effect of GATA-3 on the latter shows no constant change with time. The up-regulation of GATA-3 by pSTAT6 remains similar throughout the week, while repression by pSTAT1 only becomes significant on day 3.

Linear regression analysis predicts that several self-reinforcing loops are involved in the regulation of total STAT expression: pSTAT1 and pSTAT6 up-regulating STAT1 and STAT6, respectively, and T-bet activating STAT1 and STAT4 expression. In contrast, pSTAT4 is predicted to inhibit the expression of both Th1-specific signal transducers STAT1 and STAT4. Mutually repressive mechanisms described by the best-fitting models include the down-regulation of STAT6 by pSTAT1 and of STAT4 by GATA-3.
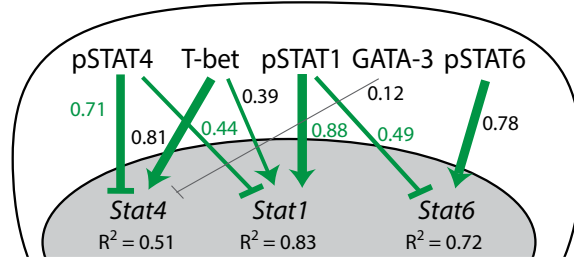
Figure 3.11: Observed ways of STAT regulation. Graphical representation of the best linear models describing the effect of pSTAT1, pSTAT4, pSTAT6, T-bet and GATA-3 on STAT1, STAT4 and STAT6 expression. Grey arrows represent previously described interactions, green arrows hitherto undescribed mechanisms. Numbers show the absolute value of strength of regulation on day 4; 0 stands for non-significant effect.

3.3 Master transcription factors expression is predictive for the cytokine recall response

We determined how cytokine signals influence transcription factor expression via specific signal transducers, the STAT proteins. The next step was to study how this affected the function of Th cells, which is carried out by secretion of effector cytokines. In chapter 2, we observed similarities in the expression patterns of T-bet and/or GATA-3 and that of IFN-γ, TNF-α, IL-2, IL-4 and IL-13, while the pattern of IL-10 expression seemed unique. We thus studied the relationship between master transcription factors and the cytokines produced by the differentiated cells upon restimulation using linear regression analysis. Binding of the master transcription factors to cytokine genes has been shown, as well as contributions of the STAT proteins. However, in the setup used, cytokine production was measured after strong antigen stimulus in the absence of polarising cytokines and hence without acute STAT activation. We thus constructed linear models explaining the fraction of cytokine producers as functions of T-bet and/or GATA-3 and omitted pSTAT contribution. The fraction of cells expressing each cytokine could thus be explained by a family of four linear functions of either T-bet, GATA-3, both of them or none of them.

$$F_i = \alpha_{1,i} \text{ T-bet} + \alpha_{2,i} \text{ GATA-3}$$

where F is the fraction of producers of cytokine i for $i = $ IFN-γ, IL-4, IL-13, TNF-α, IL-2, IL-10 and allowing each $\alpha_{j,i} = 0$.

The best model among a family was selected, as described before, according the R^2, AIC values and significance of the parameters of each fit, and standard diagnostics were checked (Supplementary Figure 7.7). We found that IFN-γ and IL-4 were well explained as functions of only T-bet or GATA-3, respectively
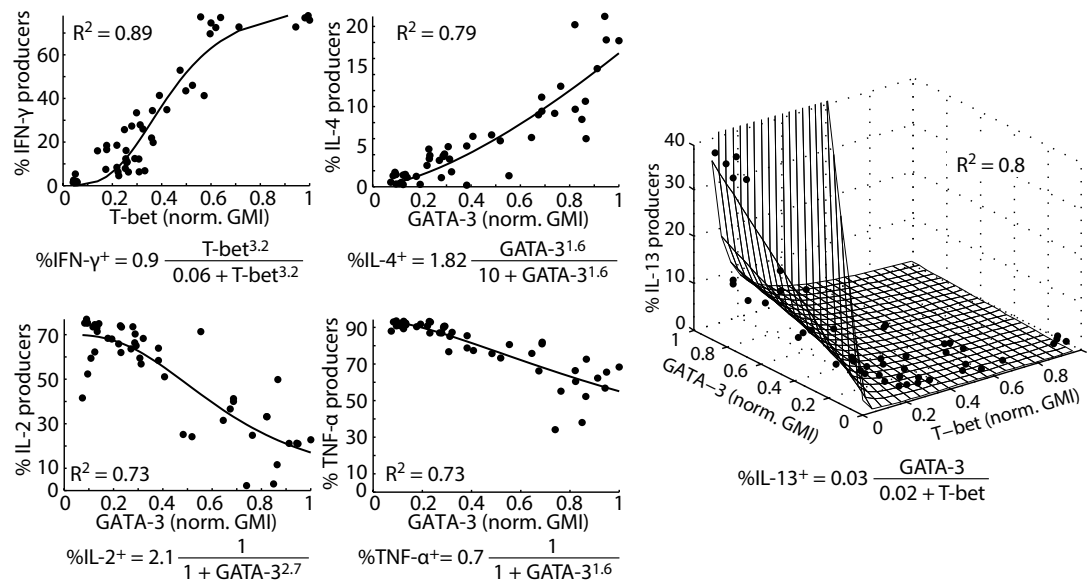
Figure 3.12: Cytokine expression can be expressed as functions of T-bet and GATA-3 expression. FACS-sorted naive IFN-γ- and IL-4-competent CD4 T cells were activated with APCs and GP$_{61-80}$ peptide under the indicated conditions. IL-12 and IL-4 were titrated against each other. On day 5, T-bet and GATA-3 expression were measured and the cells were restimulated with PMA and ionomycin in the presence of brefeldin A for 3 hours before cytokines were stained intracellularly. Nonlinear functions expressing cytokine producers as function of T-bet and/or GATA-3 were derived based on linear regression analysis and fitted to the data.

($R^2 = 0.83$ and $R^2 = 0.71$), suggesting no direct role of the adverse factor in the regulation of their recall response by antigen alone. By contrast, IL-13 is better accounted for by both MTFs ($R^2 = 0.49$), correlating positively with GATA-3 and negatively with T-bet. Both TNF-α and IL-2 are explained well as negative functions of GATA-3 ($R^2 = 0.79$ and 0.72, respectively). Of all cytokines, the IL-10 recall response was least well explained by the expression levels of the MTFs ($R^2 = 0.44$). The linear fits could be further improved by empirically choosing appropriate nonlinear functions based on the results of the linear fits, with the exception of IL-10 (Figure 3.12). The nonlinear functions suggest cooperativity (i.e., Hill coefficients larger than 1) for the activating and inhibiting actions of both master transcription factors.

The linear analysis of cytokine expression upon recall response show that quantitative differences in T-bet and GATA-3 expression levels are highly predictive for the fraction of cytokine-expressing cells upon antigen reencounter. The expression of the Th1 and Th2 signature cytokines IFN-γ and IL-4 were strongly positively correlated to T-bet and GATA-3, respectively, while TNF-α and IL-2 responded negatively to GATA-3. IL-13 was correlated positively to GATA-3 and negatively to T-bet.

3.4 Conclusion

Linear regression analysis of time-resolved dose-response data allowed us to reconstruct the signalling network that governs master transcription factor expression in Th cells differentiated with mixed Th1-Th2 stimuli. Specifically, we predicted novel functional roles for pSTAT1 as well as pSTAT4 and found numerous regulatory mechanisms acting on STAT1, STAT4 and STAT6 expression, thus mediating rewiring of the signalling pathways during differentiation. Furthermore, the expression levels of both T-bet and GATA-3 were correlated to the amount of phosphorylated pSTAT1, pSTAT4 and pSTAT6, which in turn were determined by cytokine signals and total STAT expression − the latter being regulated by both the pSTATs and the master transcription factors. Although there were positive cross-effects at the STAT activation level (STAT1 and STAT4 phosphorylation by IL-4), all interactions between the Th1 and the Th2 players were negative at the layer of transcription factor regulation. The regulatory mechanisms involving only Th1 or Th2 factors were enhancing ones, except for negative pSTAT4 effects on both STAT1 and STAT4 expression. In summary, the amount of polarising cytokines present during differentiation determines the extent of STAT phosphorylation, total STAT expression, and master transcription factor expression in the cells, which in turn determines the probability of cytokine expression by a cell during an immune response, linking primary differentiation conditions to functional output.

Chapter 4

Dynamical model

In the previous chapters, we defined the topology of the network of interactions between the cytokines IFN-γ, IL-12 and IL-4, the downstream transcription factors STAT1, STAT4 and STAT6, and the master transcription factors T-bet and GATA-3, using linear regression analysis on quantitative, time-resolved dose-response data (c.f. Figure 5.1). The network depicts positive feedback loops inside the Th1 and Th2 modules (i.e., up-regulation of STAT1 and STAT4 by T-bet, auto-activation of STAT1 and STAT6). Between the two modules, negative interactions dominate. The Th1 STATs play a major role in inhibiting Th2 differentiation: both STAT1 and STAT4 repress GATA-3 in a T-bet independent manner, and STAT1 also down-regulates STAT6. Negative regulation of the Th1 pathway by the Th2 module is mediated by GATA-3 inhibiting T-bet expression. However, positive cross-talk in the form of STAT1 and STAT4 phosphorylation downstream of IL-4 was also observed, as well as negative feedback of STAT4 on both STAT1 and STAT4 expression inside the Th1 pathway.

The linear regression approach was very helpful for the determination of correlations between the different protein species considered, but could not explain the kinetics of protein expression during primary differentiation. Furthermore, as several factors correlated strongly with each other (e.g., T-bet with pSTAT1 and pSTAT4, pSTAT1 with IFN-γ and IL-12, and pSTAT4 with IL-12) and correlation does not inform about causality, linear modelling could not determine unambiguously which of the correlated factors was responsible for a given effect (e.g., is pSTAT1 or T-bet responsible for the up-regulation of STAT4, or does the correlation result from another mechanism not considered?). In this chapter, we developed a dynamical model that relies on assumptions about causal relationships. By confronting this model, based on ordinary differential equations (ODEs), with the time-resolved data, we will gain further insight into the topology and dynamics of the network.

4.1   The fitted dynamical model is able to reproduce the kinetics of STAT phosphorylation as well as STAT, T-bet and GATA-3 expression

The development of a quantitatively predictive dynamical model requires the description of the network of interactions being modelled as well as data to estimate the model's parameter. The topology of the Th1/Th2 signalling network derived from the linear regression analysis was described in Chapter 3, while the experiments presented in Chapter 2 provide quantitative data about STAT phosphorylation as well as STAT and master transcription factor expression. We thus proceeded with the development, fitting and testing of a dynamical model of Th1/Th2 cell differentiation.

The network topology described in the previous chapter was used to develop a simple dynamical mechanistic model. The model is based on ordinary differential equations and explains the expression of T-bet, GATA-3, STAT1, STAT4 and STAT6 as well as the phosphorylation of the STATs, taking the polarising cytokine concentrations as inputs. To account for receptor saturation and desensitization, the logarithms of the concentrations are used. In the model used from here on, the cytokine concentrations are assumed to decrease with time due to consumption and degradation according to hyperbolic tangent functions (Figure 4.1). :

$$[\text{IFN-}\gamma] = C_{\text{IFN-}\gamma} \cdot (1 - \tanh(t - T_{\text{IFN-}\gamma})) \tag{4.1}$$

$$[\text{IL-12}] = C_{\text{IL-12}} \cdot (1 - \tanh(t - T_{\text{IL-12}})) \tag{4.2}$$

$$[\text{IL-4}] = C_{\text{IL-4}} \cdot (1 - \tanh(t - T_{\text{IL-4}})) \tag{4.3}$$

The Cs are the logarithm of the starting cytokine concentrations, t the time in days, and the Ts are the half-lives of the cytokines.

The phosphorylation of the STATs is fast (minutes) compared to the timescale of the experiment (5 days). Therefore, we express the pSTATs as algebraic functions of cytokine concentrations, assuming a linear relationship:

$$\text{pS1} = (\alpha_1[\text{IFN-}\gamma] + \alpha_2[\text{IL-12}] + \alpha_3[\text{IL-4}]) \cdot \text{S1} \tag{4.4}$$

$$\text{pS4} = (\alpha_4[\text{IL-12}] + \alpha_5[\text{IL-4}]) \cdot \text{S4} \tag{4.5}$$

$$\text{pS6} = (\alpha_6[\text{IL-4}]) \cdot \text{S6} \tag{4.6}$$

The amounts of phosphorylated STATs are represented by pS1, pS4 and pS6, while total STAT protein expression levels are represented by S1, S4 and S6. The αs the strength of the cytokine effects on STAT phosphorylation.
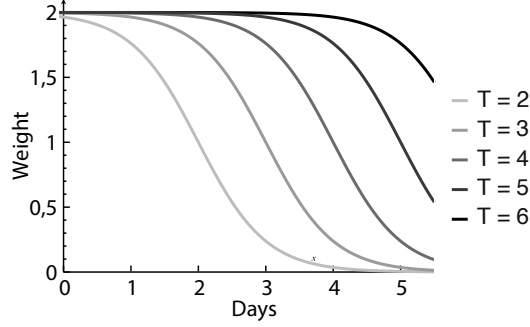
Figure 4.1: Different possible kinetics for the cytokine concentrations. The functions $(1 - \tanh(t - T))$ with different T values used to describe the kinetics of cytokine concentration.

The transcription factor protein expression was modelled by a system of five ordinary differential equations, one for each protein. The pSTATs were assumed to act in concert with antigen signalling (as differentiation does not occur in the absence of the latter), so that an equation describing the dynamics of the antigen stimulus was added to the system:

$$\text{Ag} = \left(1 - e^{(-t)}\right) \cdot e^{(-l_2 \cdot t)} \tag{4.7}$$

$$\dot{\text{S1}} = \frac{B_{\text{S1}} + \beta_1 \cdot \text{T} + \beta_2 \cdot \text{pS1} \cdot \text{Ag}}{1 + \beta_3 \cdot \text{pS4} \cdot \text{Ag}} - \delta_{\text{S1}} \cdot \text{S1} \tag{4.8}$$

$$\dot{\text{S4}} = \frac{B_{\text{S4}} + \beta_4 \cdot \text{T}}{1 + \beta_5 \cdot \text{pS4} \cdot \text{Ag} + \beta_6 \cdot \text{G}} - \delta_{\text{S4}} \cdot \text{S4} \tag{4.9}$$

$$\dot{\text{S6}} = \frac{B_{\text{S6}} + \beta_7 \cdot \text{pS6} \cdot \text{Ag}}{1 + \beta_8 \cdot \text{pS1} \cdot \text{Ag}} - \delta_{\text{S6}} \cdot \text{S6} \tag{4.10}$$

$$\dot{\text{T}} = \frac{B_{\text{T}} + \beta_9 \cdot \text{pS1} \cdot \text{Ag} + \beta_{10} \cdot \text{pS4} \cdot \text{Ag}}{1 + \beta_{11} \cdot \text{G}} - \delta_{\text{T}} \cdot \text{T} \tag{4.11}$$

$$\dot{\text{G}} = \frac{B_{\text{G}} + \beta_{12} \cdot \text{pS6} \cdot \text{Ag}}{1 + \beta_{13} \cdot \text{pS1} \cdot \text{Ag} + \beta_{14} \cdot \text{pS4} \cdot \text{Ag}} - \delta_{\text{G}} \cdot \text{G} \tag{4.12}$$

S1, S4, S6, T and G represent STAT1, STAT4, STAT6, T-bet and GATA-3 protein expression, respectively. Ag represents the antigen stimulus, which increases with time constant 1 per day and decreases with time constant l_2 (Figure 4.2). pS1, pS4 and pS6 represent the phosphorylated forms of STAT1, STAT4 and STAT6, respectively. Each protein is produced with a basal rate B_i and degraded with a rate δ_i. In addition to that, the positive and negative regulatory mechanisms described by the linear regression analysis are added to the production rate with weights β_j. For simplicity, we only write dynamic equations for the protein concentrations and not the respective mRNAs; due to their short lifetime, mRNA
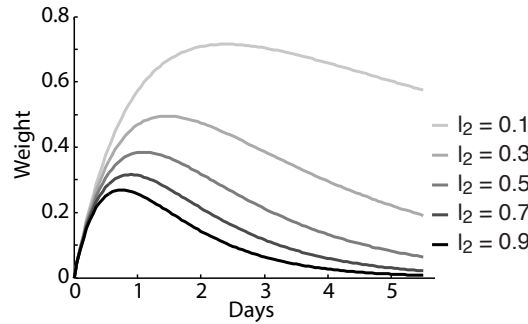
Figure 4.2: Different possible kinetics for the antigen signal. The functions $(1 - e^{(-t)}) \cdot e^{(-l_2 \cdot t)}$ with different l_2 values used to describe the kinetics of antigen signalling.

concentrations will rather rapidly adapt to the given stimulus and hence assume quasi-steady states.

	STAT1	STAT4	STAT6	T-bet	GATA-3
B	0.21	0.24	0.21	0.01	0.11

Table 4.1: Basal production rates

The model written above is composed of an algebraic part (equations 4.1 to 4.7) and a differential part (equations 4.8 to 4.12) and includes all the connections derived from the linear regression analysis; we assumed that all regulators acted in an additive manner. We proceeded to fit this model to the normalised titration data in two steps: first, we fitted the algebraic part to determine the best α and T values to model the pSTAT intensities; the T and α parameters were estimated using Wolfram Mathematica 10 independently of the differential equation part of the model describing transcription factors expression. The pSTAT intensities pS1, pS4 and pS6 were fitted to the corresponding data values from the IFN-γ, IL-12 and IL-4 titrations, taking the experimental values for the total STAT intensities S1, S4 and S6 as inputs.

Second, we fitted the parameters of the ODE system describing transcription factor expression with Matlab using the Data2Dynamics (D2D) framework[185,186]. The basal rates B_i were fixed as to obtain the initial conditions derived from the data (Table 4.1). Equations 4.1 to 4.6 with the previously fitted values for the α and T parameters were used as inputs. l_2, the δ and the β parameters were estimated. Furthermore, we fitted an error model to the data constituted of an absolute error E_{abs} and a relative error E_{rel} to the ODE model.

We first considered the algebraic part of the model; the best-fit parameter values and their confidence intervals are shown in Table 4.2. The best-fit values for the T parameters show that cytokine degradation and consumption play a minor role in our time scale: only the IFN-γ concentration decreases significantly before
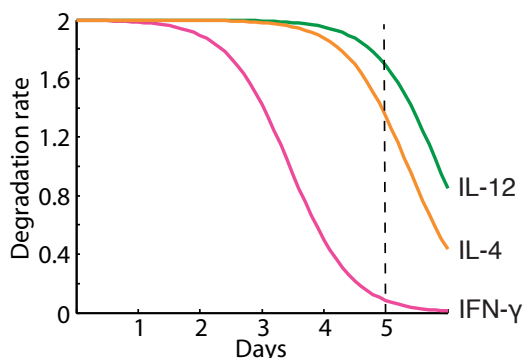
Figure 4.3: Cytokine degradation is slow with half-lifes around 5 days. The functions $(1 - \tanh(t - T_i))$ with the fitted values for $T_{\text{IFN-}\gamma}$, $T_{\text{IL-12}}$ and $T_{\text{IL-4}}$ describe the degradation and consumption of IFN-γ, IL-12 and IL-4 during the five days of culture.

day 5 (Figure 4.3). The values for the α parameters are consistent with our linear regression analysis: The greatest contributions come from the canonical cytokine upstream from the STAT (i.e., IFN-γ for pSTAT1, IL-12 for pSTAT4 and IL-4 for pSTAT6), with smaller additions from IL-12 and IL-4 for pSTAT1 and from IL-4 for pSTAT4 (Table 4.2).

Effect on	Effect of	Parameter	Value	Lower bound	Upper bound
IFN-γ	Time	$T_{\text{IFN-}\gamma}$	3.45	3.17	3.72
IL-12	Time	$T_{\text{IL-12}}$	5.85	5.37	6.33
IL-4	Time	$T_{\text{IL-4}}$	5.36	5.16	5.57
pSTAT1	IFN-γ	α_1	0.34	0.3	0.39
pSTAT1	IL-12	α_2	0.21	0.16	0.25
pSTAT1	IL-4	α_3	0.22	0.17	0.26
pSTAT4	IL-12	α_4	0.49	0.45	0.53
pSTAT4	IL-4	α_5	0.13	0.1	0.17
pSTAT6	IL-4	α_6	0.54	0.52	0.56

Table 4.2: Parameter values for the best fit of the model describing STAT phosphorylation

Having determined the values of the parameters of the algebraic part of the model using the experimental values for the total STAT, we could input the functions describing cytokine concentration and pSTAT intensity in the complete model and estimate the parameters regulating the expression of T-bet, GATA-3, STAT1, STAT4 and STAT6. To do so, we used only the starting cytokine concentrations as input values and fitted STAT1, STAT4, STAT6, T-bet and GATA-3 expression to the corresponding normalised experimental values from the kinetic titration experiments. The fitting procedure was performed 300 times with different starting values for the parameters generated by latin hypercube sampling[187] to ascertain that a global minimum was found (Supplementary Figure 4.4a); the 95% confidence intervals and the identifiability of the parameters giving the best fit were

Effect on	Effect of	Parameter	Value	Lower bound	Upper bound
Antigen	Time	l_2	0.53	0.44	0.61
Stat1	T-bet	β_1	1.27	1.02	1.57
Stat1	pSTAT1	β_2	5.65	4.65	6.82
Stat1	pSTAT4	β_3	11.8	9.88	14.06
Stat4	T-bet	β_4	0.27	0.14	0.42
Stat4	pSTAT4	β_5	2.1	0.44	4.28
Stat4	GATA-3	β_6	0.83	0.38	1.52
Stat6	pSTAT6	β_7	1.51	1.21	1.86
Stat6	pSTAT1	β_8	1.31	0.59	2.16
Tbx21	pSTAT1	β_9	3.95	3.27	4.89
Tbx21	pSTAT4	β_{10}	3.26	2.66	4.07
Tbx21	GATA-3	β_{11}	6.68	4.90	9.47
Gata3	pSTAT6	β_{12}	4.98	3.93	6.37
Gata3	pSTAT4	β_{13}	9.58	6.09	14.58
Gata3	pSTAT1	β_{14}	9.13	5.06	13.58
Stat1	Degradation	δ_{S1}	1.56	1.41	1.73
Stat4	Degradation	δ_{S4}	0.46	0.36	0.55
Stat6	Degradation	δ_{S6}	0.45	0.42	0.49
Tbx21	Degradation	δ_{T}	0.27	0.18	0.38
Gata3	Degradation	δ_{G}	0.46	0.41	0.51
All	Absolute error	E_{abs}	0.04	0.03	0.04
All	Relative error	E_{rel}	0.31	0.29	0.33

Table 4.3: Parameter values for the best fit of the model describing TF regulation

estimated using the profile likelihood[188,189]. The resulting best-fit parameters and their confidence intervals are shown in Table 4.3. All fitted parameters were uniquely defined as determined by the profile likelihood estimations (Figure 4.4b). The model with the best-fit parameter values allowed a good reproduction of the global titration data, as illustrated in Figure 4.5 by scatter plots of the data points versus the model predictions.

Having obtained uniquely defined parameters by fitting the model to the data, we evaluated the relevance of each interaction in the model based on the linear regressions. In order to do this, we compared the results of the fit of the complete model to the fits of all possible models lacking one specific interaction (defined through a regulator and its target) using the AIC (Table 4.4). Leaving out any interaction resulted in a significantly worse fit of the model to the data (ΔAIC > 2).

To determine if the dynamical model was able to reproduce the dynamical behaviour of the system, we selected different conditions for which to compare the model to the data visually, as the large number of conditions made inspection of the whole dataset difficult. We simulated the phosphorylation kinetics of the three STATs in response to IFN-γ, IL-12 and IL-4 separately (Figure 4.6). The model was able to reproduce the dynamics of STAT phosphorylation (the total STAT values
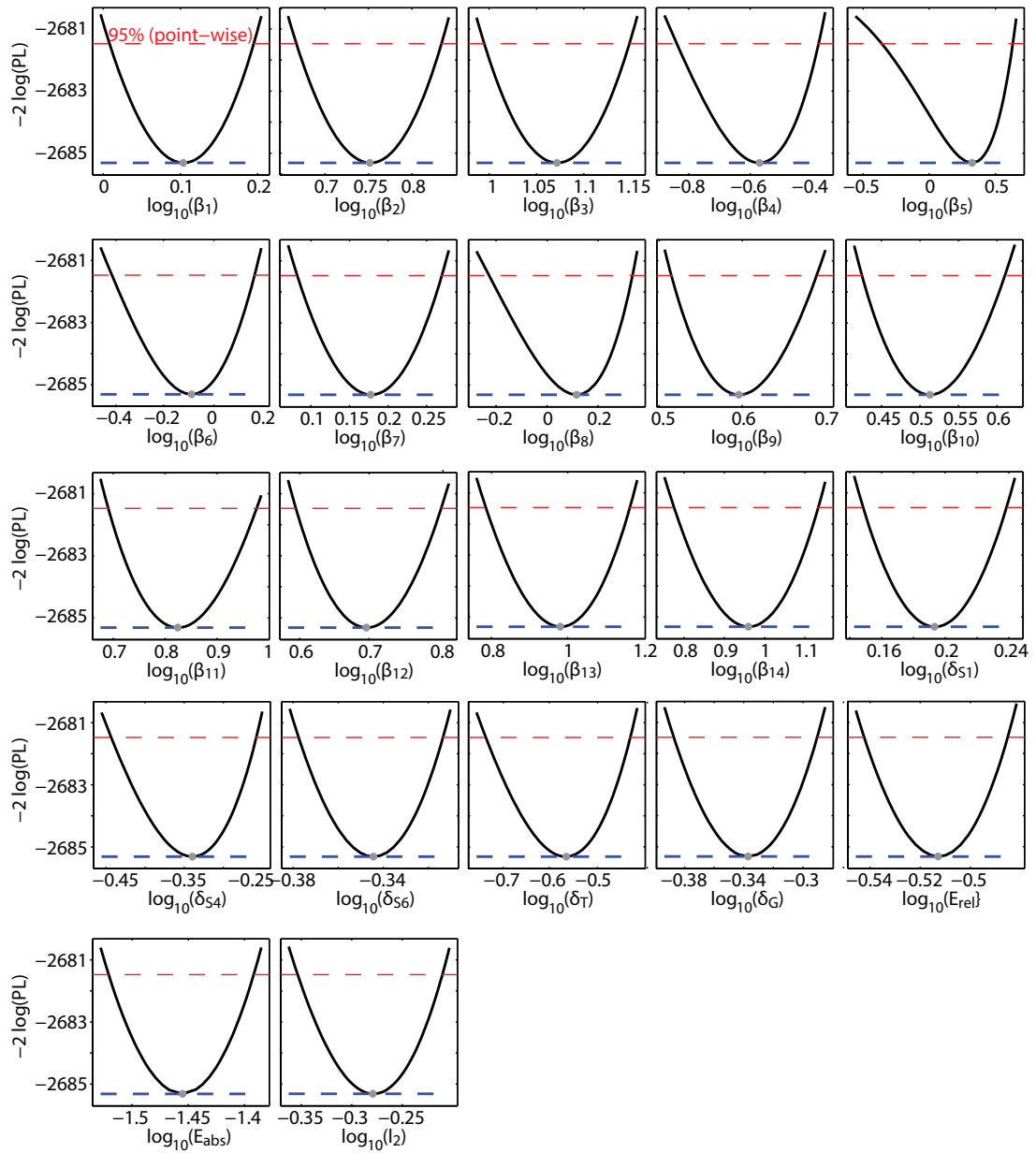
Figure 4.4: Fitting the model leads uniquely defined parameters. Profile likelihood estimation of the parameter values from the differential equation part of the model.
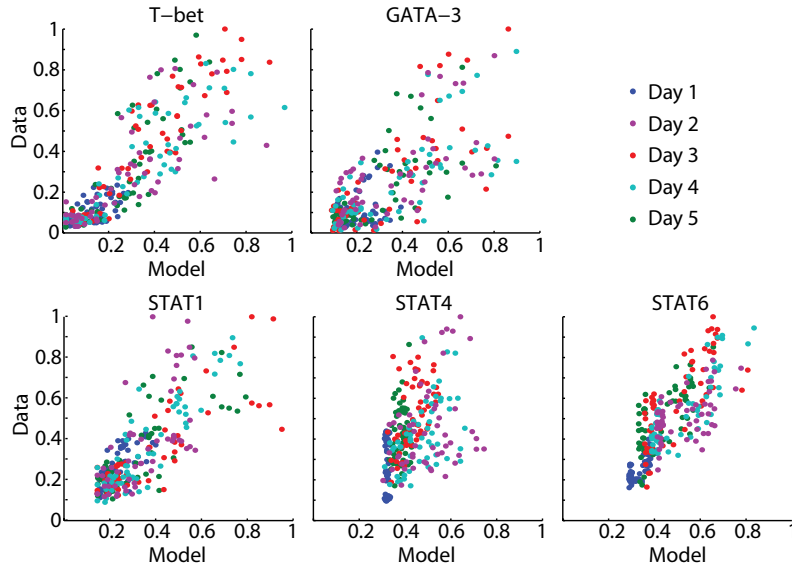
Figure 4.5: The data correlates to the values generated by the fitted model. The normalised data for T-bet, GATA-3, STAT1, STAT4 and STAT6 expression from day 1 to day 5 in all conditions was plotted against the corresponding values computed by the fitted model.

Target	T-bet	T-bet	T-bet	GATA-3	GATA-3	GATA-3
Regulator	STAT1	STAT4	GATA-3	STAT1	STAT4	STAT6
ΔAIC	874.4	463.7	320.7	75.4	62.4	1193.1

Target	STAT1	STAT1	STAT1	STAT4	STAT4	STAT4	STAT6	STAT6
Regulator	STAT1	STAT4	T-bet	STAT4	T-bet	GATA-3	STAT1	STAT6
ΔAIC	164.7	458.1	207.7	3.5	18.3	16.1	11.4	206.9

Table 4.4: Difference in the AIC value of all possible models lacking one interaction to the complete model

generated by the model were used for the simulation, and not the data values used for the fitting procedure of the algebraic part of the model); the simulated and experimental responses of all three pSTATs to the amounts of IFN-γ, IL-12 and IL-4 used in 'classical' Th1 and Th2 cultures (i.e., 10 ng/ml IFN-γ, 5 ng/ml IL-12 and 10 ng/ml IL-4) are depicted in Figure 4.6a.

In addition, we chose Th1, Th2 and hybrid Th1/2 conditions and considered transcription factor regulation from day 1 to day 5, and compared the simulated model values to the experimental values (Figure 4.6b). The up-regulation of T-bet and GATA-3 was accurately reproduced, although GATA-3 was overestimated in the classical Th2 conditions. STAT1 and STAT6 dynamics could also be explained by the model (Figure 4.6c). STAT4 was more problematic, consistently with the low R^2 values from the linear analysis.
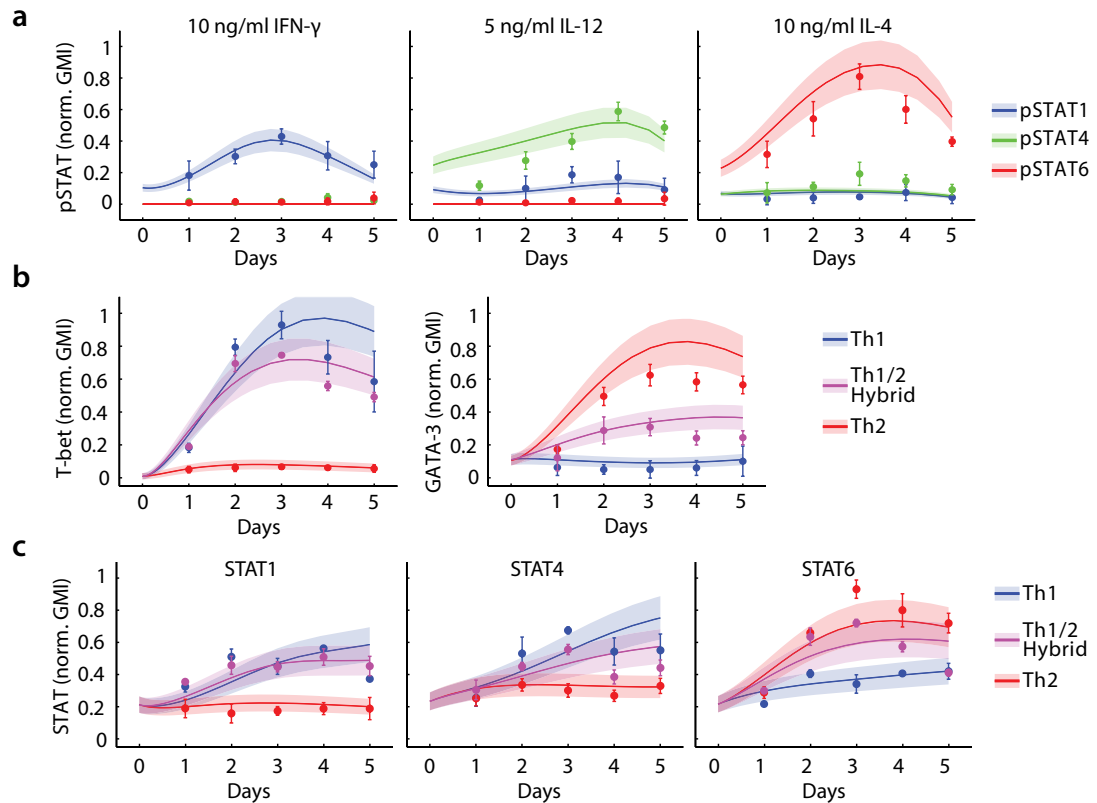
Figure 4.6: The model can reproduce the time courses of up-regulation of the studied factors. a Phosphorylation of STAT1 (blue), STAT4 (green) and STAT6 (red) downstream of IFN- (left), IL-12 (middle) and IL-4 (right) as predicted by the model (solid lines) and as seen in the data (dots). **b** up-regulation of T-bet (left) and GATA-3 (right) during differentiation under Th1 (blue), Th2 (red) and hybrid Th1/2 (purple) conditions as predicted by the model (solid lines) and as seen in the data (dots). **c** up-regulation of STAT1 (left), STAT4 (middle) and STAT6 (right) during differentiation under Th1 (blue), Th2 (red) and hybrid Th1/2 (purple) conditions as predicted by the model (solid lines) and as seen in the data (dots). The dots represent the normalised geometric mean indices SD, and the model curves are shown with the error margins fitted to the data.

A dynamical model based on the network topology derived from linear regression analysis was able to describe and quantitatively reproduce the dynamical behaviour of its main components, in our case, pSTAT1, pSTAT4, pSTAT6, T-bet, GATA-3, STAT1 and STAT6.

4.2 The dynamical model reproduces a continuum of T-bet and GATA-3 expression levels in response to graded stimuli

Classically, mutual inhibition and auto-activation motifs as present in our model are thought to give rise to a bistable system in steady state, which would lead to a digital switch between high T-bet expression and high GATA-3 expression under mixed Th1-Th2 stimuli[156], or a quadristable system allowing for an intermediate co-expressing steady state as well as a naive low expressing state (MSc dissertation, E. Pellet). However, our experiments have shown that there is a continuum of expression patterns of T-bet and GATA-3 in response to titrated cytokine amounts during differentiation (cf. Figure 2.9). Therefore, we asked which kind of steady states our parametrised model has.

To analyse the stability of our system upon differentiation in a visually intuitive manner, we considered the phase plane of T-bet and GATA-3 expression under quasi-steady state assumption for STAT1, STAT4 and STAT6, i.e., the assumption that STAT amounts did not vary anymore ($\dot{S1} = \dot{S4} = \dot{S1} = 0$, Figure 4.7) with fixed values for the pSTAT intensities. We thus reduced the system to two dimensions, T-bet and GATA-3. This approach allows to study the system at steady state and to determine the possible stable expression patterns of T-bet and GATA-3 at the end of differentiation. Using Wolfram Mathematica 10, we computed the T-bet-GATA-3 pairs of values at which GATA-3 remains stable by solving $\dot{G} = 0$ and $\dot{T} = 0$ for GATA-3 (GATA-3 nullclines, red lines), and those at which T-bet remains stable by solving for T-bet (T-bet nullclines, blue lines). Thus, the intersection of a T-bet and GATA-3 nullcline represents a steady state for T-bet and GATA-3. We also computed the vector field (blue arrows), which shows if T-bet and GATA-3 expression levels are increasing or decreasing at each point of the T-bet-GATA-3 phase plane. We titrated the input Th1 stimuli IL-12 and IFN-γ versus the Th2 stimulus IL-4 and observed the changes in the T-bet-GATA-3 phase plane.

Interestingly, our model has a unique stable steady state that moves along the axes depending on the cytokine stimuli. Thus the model shows a continuum of T-bet-GATA-3 expression patterns in response to mixed stimuli rather than division of the population between GATA-3 and T-bet-expressing cells depending on the polarising stimuli, which would become manifest by two stable steady states on the GATA-3 and T-bet axis, respectively (Figure 4.7). This was indeed what we had previously observed experimentally on day 5 of the IL-12-IL-4 cross-titration in IFN-γ-producing cells: a unimodal population expressing more T-bet in the presence of higher Th1 cytokine concentrations, and more GATA-3 in the presence of higher IL-4 concentrations (see Figure 2.9).
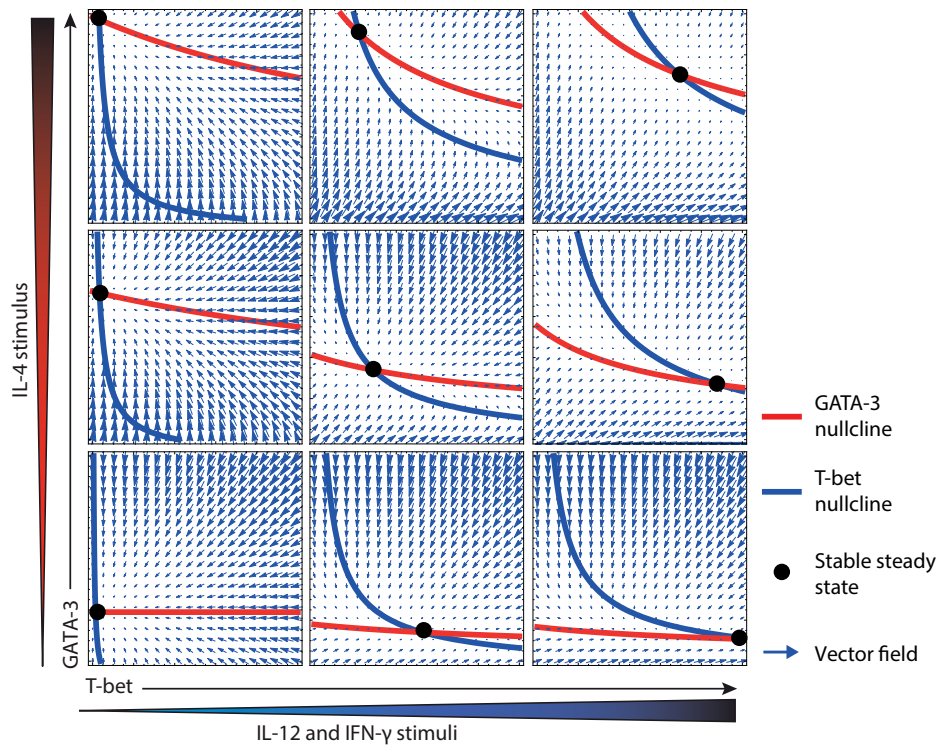
Figure 4.7: Steady-state behaviour of the dynamical model. Phase plane analysis of T-bet and GATA-3 under the assumption of quasi-steady state for all other variables. The system has only one stable steady state; its value depends on cytokine inputs and moves gradually in the T-bet-GATA-3 space.
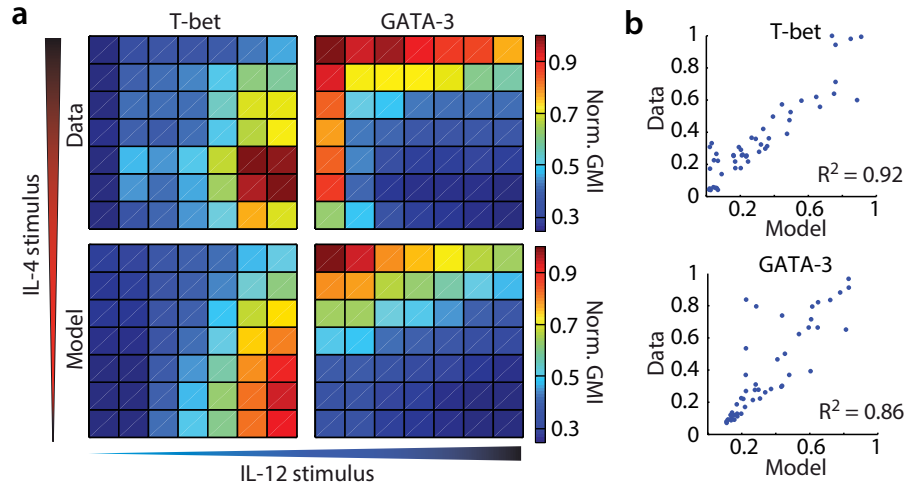
Figure 4.8: The model quantitatively reproduces independent data from an IL-12-IL-4 crosstitration. FACS-sorted naive IFN-γ- and IL-4-competent CD4 T cells were activated with APCs and GP$_{61-80}$ peptide under the indicated conditions. IL-12 and IL-4 were titrated against each other. T-bet and GATA-3 expression on day 5 as well the the values predicted by the model are shown as heat maps and dot plots.

Having determined that the dynamical model possessed only one steady state for T-bet and GATA-3 expression, we then considered the quantitative response of the model to graded mixed Th1-Th2 stimuli during differentiation in the form of the expression intensity of the master transcription factors. To do so, we simulated the results of an IL-12-IL-4 cross-titration experiment after five days of differentiation. Under these conditions, IFN-γ is produced by the Th cells (and not added in a controlled manner), we extrapolated the IFN-γ concentration based on the T-bet expression on day 5 using the model for IFN-γ production described in Chapter 3 (see Figure 3.12):

$$\%\text{IFN-}\gamma^+ = 0.9 \cdot \frac{\text{T}^{3.2}}{0.06 + \text{T}^{3.2}}$$

Figure 4.8 shows the comparison of the experimental values of T-bet and GATA-3 expression to the values predicted by the model with the best-fit parameters estimated previously. Heatmaps of T-bet and GATA-3 expression show a similar response to IL-12 and IL-4 (Figure 4.8a) between the experimental data and the model; T-bet showed, in both cases, an increase in the presence of IL-12 and IFN-γ and a decrease in the presence of IL-4, while the opposite was true of GATA-3. To verify that the model was able to quantitatively reproduce the data, we computed the correlation between the data and predicted values; the model was able to predict the expression of T-bet and GATA-3 accurately with correlation coefficients R^2 of 0.92 for T-bet and 0.86 for GATA-3 (Figure 4.8b).

The dynamical model developed in this study has a single, stable fixed point considering the quasi-steady state for T-bet and GATA-3. The value of this fixed point moves continuously along the T-bet and GATA-3 axes depending on the input cytokine stimuli. Furthermore, the model was able to reproduce accurately the continuum of T-bet and GATA-3 expression levels derived from an independent IL-12-IL4 cross-titration experiment.

4.3   The novel effects of pSTAT1 and pSTAT4 are T-bet-independent

The linear models described several novel effects of pSTAT1 and pSTAT4 on GATA-3, STAT1, STAT4 and STAT6, which were included in the dynamical model. Specifically, pSTAT1 was predicted to up-regulate STAT1 expression while repressing STAT6 and GATA-3 expression. Moreover, pSTAT4 was predicted to repress STAT1, STAT4 and GATA-3 expression. Although the selection of the best-fitting models leading to those interactions was unambiguous according to the AIC (ΔAIC > 2, see Figures 3.5, 3.8 and 3.10), the next best models also fitted the data well and often substituted one pSTAT effect for a T-bet effect, or added a T-bet effect to the selected model. For example, the best model for GATA-3 included pSTAT1, pSTAT4 and pSTAT6 as regulators (model A1), the next best models, according to the AIC, were models C7, in which T-bet replaced both pSTAT1 and pSTAT4; C3, in which T-bet replaced pSTAT4; C4, in which T-bet replaced pSTAT1 and C1, in which a T-bet effect was added to that of pSTAT1 and pSTAT4. This is due to the high correlation between pSTAT1, pSTAT4 and T-bet. It is therefore unclear how unambiguously the linear models can distinguish between T-bet-dependent and T-bet-independent effects.

To evaluate the importance of these effects in the dynamical model, we compared models corresponding to the linear model predictions with models lacking pSTAT1 (Figure 4.9a) or pSTAT4 (Figure 4.9b) effects on a specific target protein. We compared the results of the IFN-γ (for pSTAT1) or IL-12 (for pSTAT4) titrations to the predictions of the complete model (as predicted by the linear regression analysis) and of models lacking the studied effects (e.g., repression of GATA-3 by pSTAT1).

In all cases, the original model reproduced the data significantly better than an incomplete model, both qualitatively and quantitatively. The absence of a pSTAT1 effect on GATA-3 and STAT6 lead to an overestimation of both factors, while the positive correlation between STAT1 and IFN-γ was lost if pSTAT1 wasn't allowed to regulate its total expression, suggesting an important role for STAT1 in GATA-3, STAT1 and STAT6 regulation (Figure 4.9a). Similarly, the absence of pSTAT4 effects
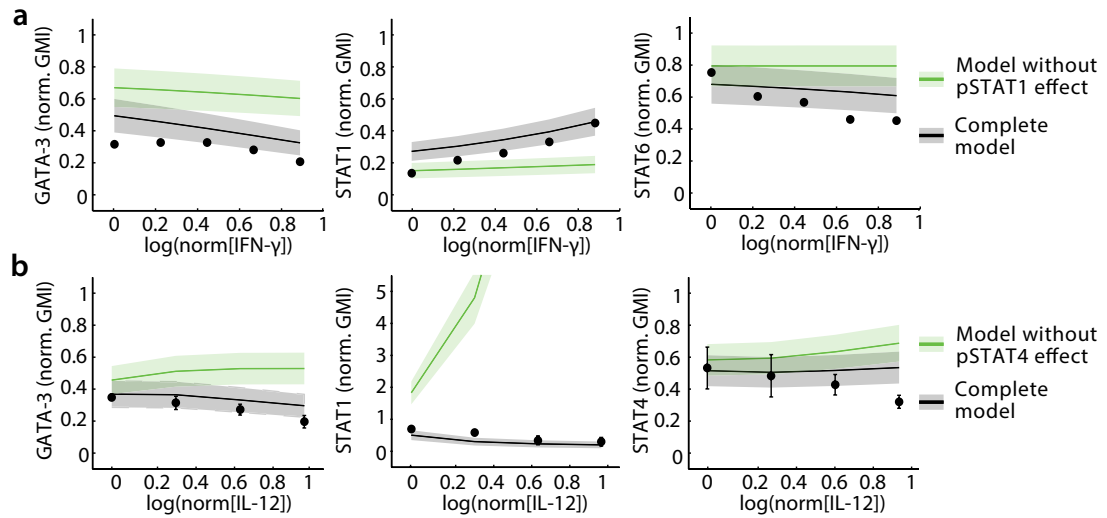
Figure 4.9: The model predicts important novel roles for pSTAT1 and pSTAT4. a Response of GATA-3, STAT1 and STAT6 to an IFN- titration in the presence of IL-12 and IL-4 as predicted by the model (solid lines) and as seen in the data (dots) for day 4. **b** Response of GATA-3, STAT1 and STAT4 to an IL-12 titration in the presence of IFN- and IL-4 as predicted by the model (solid lines) and as seen in the data (dots) for day 4. The black lines are predictions by the complete model and the green lines predictions by a model without pSTAT1 (**a**) or pSTAT4 (**b**) effects on the plotted factor. The dots represent the normalised geometric mean indices   SD, and the model curves are shown with the error margins fitted to the data.

on GATA-3, STAT1 and STAT4 caused the model to predict too high values for those factors (Figure 4.9b).

Having established that pSTAT1 and pSTAT4 had important effects on GATA-3, STAT1, STAT4 and STAT6 in our model, we went to test experimentally if those effects were indeed carried out by the predicted factor, i.e., present in a T-bet-independent manner. We first considered the role of T-bet in effects downstream of IFN- predicted to be mediated by pSTAT1. To do so, we performed IFN- titrations in *Tbx21-/-* and T-bet-competent cells and compared the responses of GATA-3, STAT6 and STAT1 to IFN- (Figure 4.10). Repression of GATA-3 and STAT6 through IFN- was fully retained in *Tbx21-/-* cells and hence T-bet-independent. STAT1 expression also remained IFN- -sensitive in the absence of T-bet, indicating direct transcriptional auto-activation of STAT1. Interestingly, even though we found T-bet to have a positive effect on STAT1, cells without T-bet had overall higher STAT1 expression, possibly as an adaptive response to the absence of T-bet. Thus, the novel effects of IFN- on both Th1 and Th2 differentiation pathways were T-bet-independent and likely mediated directly by pSTAT1.

Next we considered the predicted pSTAT4 effects downstream of IL-12, again by using *Tbx21-/-* cells to assess the role of T-bet, but additionally using *Stat4-/-* cells   as IL-12 can signal via STAT4 and STAT1. This allowed to test wether the observed effect downstream of IL-12 was indeed pSTAT4-mediated (Figure 4.11) or
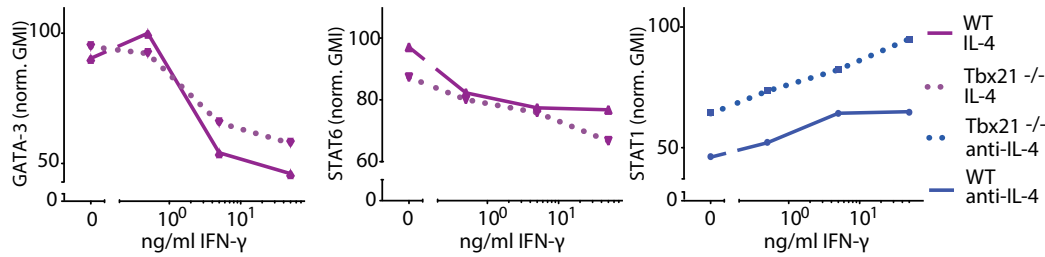
Figure 4.10: The observed regulatory effects downstream of IFN-γ are T-bet-independent FACS-sorted WT and *Tbx21-/-* naive CD4 T cells were activated with APCs and GP$_{61-80}$ peptide under the indicated conditions. IFN-γ was titrated, and transcription factor expression levels were measured on day 4 by flow cytometry. The dose response of GATA-3, STAT6 and STAT1 to IFN-γ in the absence or presence of T-bet on day 4 of differentiation are shown in Th1 conditions (blue) for the Th1 factors and in hybrid conditions (purple) for the Th2 factors. Normalised geometric mean indices are plotted.
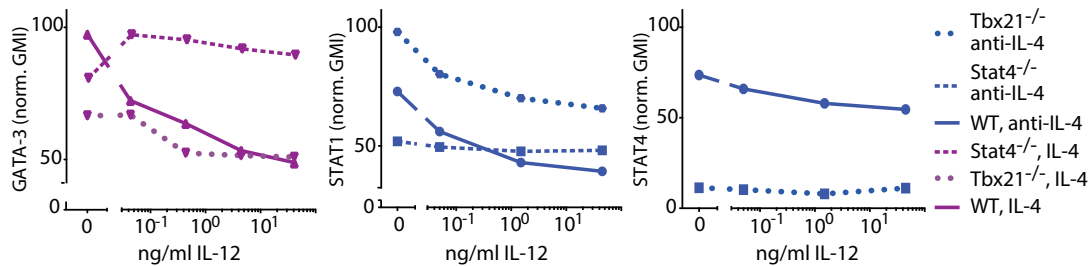
Figure 4.11: The observed regulatory effects downstream of IL-12 are T-bet-independent. FACS-sorted WT, *Tbx21-/-* and *Stat4-/-* naive CD4 T cells were activated with APCs and GP$_{61-80}$ peptide under the indicated conditions. IL-12 was titrated, and transcription factor expression levels were measured on day 4 by flow cytometry. The dose response of GATA-3, STAT1 and STAT4 to IL-12 in the absence or presence of T-bet or STAT4 on day 4 of differentiation are shown in Th1 conditions (blue) for the Th1 factors and in hybrid conditions (purple) for the Th2 factors. Normalised geometric mean indices are plotted.

depended on T-bet or the activation of STAT1 by IL-12. The repression of GATA-3 by IL-12 was independent of T-bet and STAT1 but strongly dependent on STAT4. Similarly, STAT1 repression by IL-12 did not require T-bet; in addition to that, in the absence of STAT4, the STAT1 levels were somewhat lower than in WT cells and unresponsive to IL-12. Finally, the expression of STAT4 depended strongly on T-bet: The STAT4 levels were nearly zero in the absence of the latter. Given this positive effect of T-bet, the observed decrease of STAT4 levels with the IL-12 dose in T-bet-competent cells might be attributed to an inhibitory effect of STAT4 on its own expression rather than a negative T-bet effect. Together these findings indicate that STAT4 mediates the repression of both Th1 and Th2 differentiation pathways in a T-bet-independent manner.

The dynamical model was able to explain the emergence of a continuum of mixed Th1-Th2 phenotypes in response to ambiguous polarising signals while reproducing the up-regulation dynamics of the main transcription factors. Furthermore, it predicted important, T-bet-independent roles for both pSTAT1 and pSTAT4, which we were able to confirm experimentally.

4.4 Conclusion

A simple dynamical model based on the network topology derived from linear regression analysis was developed, including all interactions predicted by the linear models in the form of ordinary differential equation for the total protein expression and linear combinations of cytokine concentration for STAT phosphorylation. The parameters of the model were estimated by fitting the latter to the titration data, resulting in all parameters being uniquely defined. The fitted model was able to reproduce the kinetics of up-regulation of the STATs, T-bet and GATA-3 as well as of phosphorylation of the STATs in response to IFN-γ, IL-12 and IL-4. Furthermore, it could replicate independent T-bet and GATA-3 protein expression data generated during an IL-12-IL-4 cross-titration. Phase plane analysis of the model for T-bet and GATA-3 at quasi-steady-state showed a single stable steady state, predicting a continuum of T-bet-GATA-3 co-expression patterns at the single-cell level in opposition to splitted T-bet- or GATA-3-expressing populations, as was observed experimentally. Finally, the model described important, T-bet independent effects of pSTAT1 and pSTAT4 on STAT1, STAT6 and GATA-3 as well as STAT1, STAT4 and GATA-3, respectively, which were confirmed experimentally using *Tbx21-/-* and *Stat4-/-* cells.

Chapter 5

Discussion

In nearly three decades of history of Th cell research, many molecular interactions that govern the functional phenotypes have been defined in great detail. Nevertheless, a surprising finding of a continuum of hybrid Th1/Th2 states[83,84,82], with hybrid cells being maintained stably in memory phase[70,82] has recently been made. Here we have developed a systematic approach for the reconstruction of regulatory networks that integrates multiple cytokine stimuli and applied it to Th cell differentiation, more specifically to Th1, Th2 and hybrid Th1/2 cell differentiation. Without investing prior knowledge about the interactions between the known key players in Th1 and Th2 differentiation, we recovered all previously well-documented regulatory interactions and uncovered numerous novel ones. We were able to develop a dynamical model of Th1-Th2 cell differentiation based on those findings that accounts for continuous levels of T-bet and GATA-3 co-expression. Here, we will summarise our findings and discuss their significance and importance for cellular differentiation and plasticity.

5.1 Summary of the Th1-Th2 signalling network

Using linear regression analysis on time-resolved dose-response data, we were able to infer the signalling network leading to hybrid Th1/2 cell differentiation. The network described in this work is summarised in Figure 5.1. Figure 5.1a shows the relationships between cytokines, transcription factor activation and transcription factor expression; Figure 5.1b recapitulates the strength of the effects on gene expression, and finally, Figure 5.1c outlines the principal ways of cytokine production regulation. This network has implications for the understanding of hybrid differentiation and the existence of a continuum between Th1 and Th2 phenotypes. We classified the interactions detected by our analysis into three types: auto-activating, mutually inhibiting and dampening interactions. While
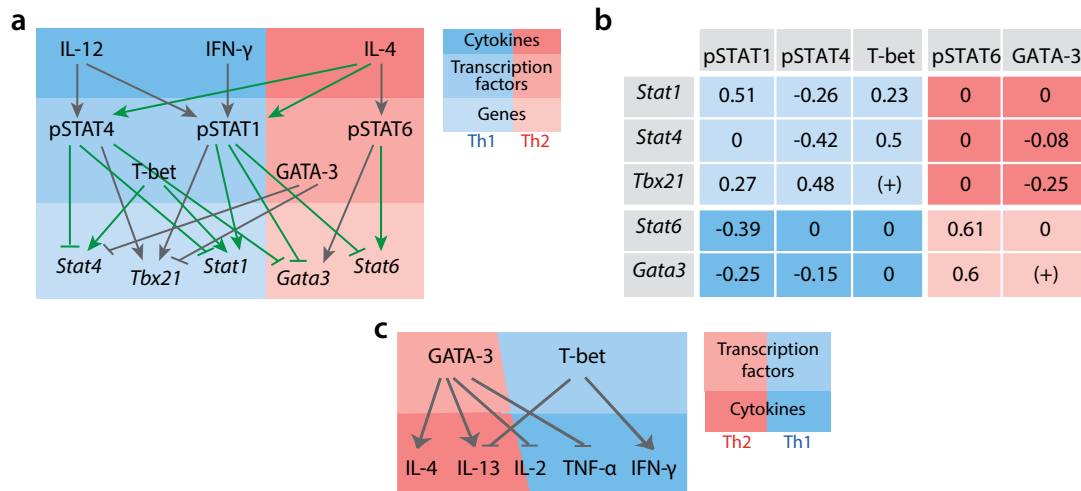
Figure 5.1: The results from the regression analysis were used to design a complete model of Th1/Th2 differentiation from the cytokine level to the MTF level and back to the cytokines. a Graphical representation of the cytokine-STAT-MTF interactions. **b** Numerical summary of the interactions between transcription factors on day 4 of culture. **c** Model of cytokine production regulation during recall response.

most of the mechanisms uncovered during the linear analysis were described in previous publications, showing that the method used to reconstruct the gene network is efficient, several novel interactions were also predicted (novel meaning here that it has not yet been described in murine Th cells). Below is a list of all interactions, their type as well as the references to previous publications.

Auto-activating interactions occur when one member of a pathway induces or up-regulates a member of the same pathway. These mechanisms may help maintain the acquired phenotype after differentiation and counteract mutual inhibitory mechanisms in the presence of adverse stimuli. The following interactions are auto-activating in our network:

In the Th1-pathway:

- The phosphorylation of STAT1 downstream of IFN-γ [171,172]
- The phosphorylation of STAT4 downstream of IL-12 [173,174]
- The phosphorylation of STAT1 downstream of IL-12 [190]
- The up-regulation of T-bet by pSTAT1 [94,191,96]
- The up-regulation of T-bet by pSTAT4 [191,96]
- The up-regulation of STAT1 by T-bet (novel)
- The up-regulation of STAT4 by T-bet (novel)
- The up-regulation of STAT1 by pSTAT1 (novel)

- The up-regulation of IFN-γ by T-bet[46]

In the Th2-pathway:

- The phosphorylation of STAT6 downstream of IL-4[175,176]
- The up-regulation of GATA-3 by pSTAT6[192]
- The up-regulation of STAT6 by pSTAT6 (novel)
- The up-regulation of IL-4 by GATA-3[48]

Mutually inhibiting interactions between the Th1 and the Th2 modules occur when one member of a pathway represses a member of the other pathway. Mutually inhibiting mechanisms limit the effects of signalling from the adverse pathway and insure that hybrid cells do not cumulate full Th1 and Th2 functions, but display reduced Th1 and Th2 functions. They are responsible for the balance between Th1-like and Th2-like phenotypic properties in hybrid cells. Those mechanisms include the following interactions in our system:

From the Th1-pathway to the Th2-pathway:

- The down-regulation of GATA-3 by pSTAT1 (novel)
- The down-regulation of GATA-3 by pSTAT4 (novel, although a negative correlation between IL-12 and GATA-3 was shown[122], the roles of STAT4 and T-bet were not studied)
- The down-regulation of STAT6 by pSTAT1 (novel)

From the Th2-pathway to the Th1 pathway:

- The down-regulation of T-bet by GATA-3 (novel)
- The down-regulation of STAT4 by GATA-3[122,121]

Dampening interactions occur when one member of a pathway down-regulates or represses a member of the same pathway, or up-regulates a member of the other pathway. Those mechanisms might support the development of hybrid cells by putting a break on one pathway to allow adverse signalling to act and also prevent over-activation of the cells in continued presence of cytokine signals. In our network, they include:

In the Th1-pathway:

- The down-regulation of STAT1 by pSTAT4 (novel)
- The down-regulation of STAT4 by pSTAT4 (novel)

In the Th2-pathway:

- The phosphorylation of STAT1 downstream of IL-4 (novel, described in human colorectal cell lines[193], in CD8 T cells[194] and in Th2, but not Th1 cells[195])

- The phosphorylation of STAT4 downstream of IL-4 (novel, described in NK cells[196])

Interestingly, an important part of the mutual inhibition was mediated by the STATs and not the master transcription factors themselves. Indeed, while GATA-3 repressed T-bet and STAT4, the repression of the Th2-pathway was carried out by pSTAT1 and pSTAT4 and was thus transient, as the STATs do not remain phosphorylated long after removal of the stimuli (e.g., half-life of pSTAT1 $< 2\text{h}$[197]). While a negative effect of IL-12 signalling on GATA-3 expression was described[122], this predates the discovery of T-bet as a Th1 master transcription factor, and thus did not directly imply STAT4, as our work now clarified. The predominant role of phosphorylated STAT protein over master transcription factors in regulating the expression of the key Th1 and Th2 proteins explains how a continuum of graded T-bet and GATA-3 expression is possible: while direct inhibition and auto-activation of T-bet and GATA-3 would result in a system having at most four possible stable expression patterns (corresponding to the naive, Th1, Th2 and hybrid phenotype), the transient nature of the STAT signals makes them rheostats in the Th1/Th2 system by changing the position of the single stable steady state in function of the cytokine inputs. This helps explain how a continuum of intermediate phenotypes can emerge in the presence of mutual repression.

5.2   STAT activation

STAT signalling is not only essential to the immune system, but is involved in several developmental processes and oncogenesis (e.g., STAT1 plays a role in mammary gland development[198] and gliomas[199]). Over 40 different factors can activate STAT signalling pathways, thus regulating processes such as apoptosis, differentiation and proliferation[200]. The study of quantitative STAT regulation in response to different cytokines is thus not only relevant to the Th cell differentiation pathways considered in this study. The analysis of STAT phosphorylation downstream of the studied cytokines led to several interesting observations: the long maintenance of STAT phosphorylation during a cell differentiation process, the presence of several cross-activations outside the canonical pathways and even between the Th1 and Th2 pathways, and the absence of negative effects directly correlating to specific cytokine signals.

The kinetics of STAT phosphorylation allow for a rewiring of the signalling network

We observed that, although transient, STAT phosphorylation was long-lasting in physiological conditions, in contrast to the prevailing paradigm in signal transduction research studying short-term signalling dynamics (over at most a few hours[199]). As phosphorylation and de-phosphorylation of STAT molecules are fast processes and thus pSTATs have short half-lives (e.g., half-life of pSTAT1 < 2h in Daudi B lymphoblastoid cells[197]), this is very likely the result of continuous phosphorylation in the presence of cytokines. The setup we used for Th cell differentiation included the incubation of naive cells directly after their *ex vivo* isolation until the end of the experiment five days later. Under such conditions, the peak of STAT1, STAT4 and STAT6 phosphorylation downstream of IFN-γ, IL-12 and IL-4, respectively, could be observed between day 3 and day 4. Furthermore, phosphorylation was unimodal among the cell population during the whole differentiation week, indicating that all cells were actively transducing the cytokine signals during that time. This prolonged phosphorylation period could be relevant for several aspects of Th cell differentiation: it could allow a cell to respond differentially to a short cytokine exposure than to a prolonged exposure, minimising unwanted differentiation or activation of the immune cells (e.g., growth inhibition by pSTAT1[201]); it could also allow the active STATs to act as transcription factors for longer periods, thus fine-tuning the regulation of their target genes, specifically the *Stat* genes themselves, leading to rewiring of the signalling network already during differentiation and modulating the cellular response.

The starting and ending time points of STAT activation seemed to be controlled by several factors, including cytokine availability and cytokine receptor expression. Indeed, regulation of cytokine receptors takes place during differentiation (Supplemental Figure 7.2). The β2 chain of the IL-12 receptor has a very low expression in naive cells, is up-regulated by IFN-γ signalling and repressed by IL-4 signalling[202,122,123] (Supplemental Figure 7.2a). We found IL12Rβ2 to be up-regulated strongly in the presence of IFN-γ between day 1 and day 3; which correlates with the intensity of STAT4 phosphorylation downstream of IL-12: STAT4 activation peaks at a later time than STAT1 and STAT6 activation. The IFN-γ receptor α chain is down-regulated during Th1 differentiation[203,204], which could explain the absence of STAT1 phosphorylation in Th1 cells at the end of differentiation, when both pSTAT4 and pSTAT6 can be observed. Furthermore, as an important role for STAT1 signalling during Th1 differentiation is the up-regulation of components of the IL-12 signalling pathway, strong STAT1 signalling is not needed anymore for Th1 differentiation once Th cells are fully IL-12 responsive. As STAT1 has been shown to exert growth inhibition downstream of IFN-γ[201], limiting STAT1

phosphorylation could be crucial for obtaining an effective immune response. The α chain of the IL-4 receptor is strongly up-regulated in the presence of IL-4[205] (Supplemental Figure 7.2b), making GATA-3-expressing cells more responsive to subsequent IL-4 signals than their Th1 counterparts and thus contributing to the maintenance of GATA-3 expression. These facts are consistent with STAT regulation by pSTATs and participate in the rewiring of the signalling network, as will be discussed in more details below.

Non-canonical effects on STAT phosphorylation are positive and can favour the opposite pathway

As mentioned above, several non-canonical inductions of STAT phosphorylation by cytokines were visible in our data: the activation of STAT1 downstream of IL-12[190], and the activation of STAT1 and STAT4 downstream of IL-4. According to our model, IFN-γ was responsible for the activation of STAT1 during the first two days of differentiation, while IL-12's (lesser) effect was most visible on days 3 and 4. Thus, IFN-γ and IL-12 could be acting additively to achieve the required amount and duration of STAT1 activation for differentiation in the Th1 direction, potentially minimising the inhibitory effect of STAT1 on cell proliferation. The effect of IL-4 was more surprising, as its canonical pathway through STAT6 induces Th2 differentiation. The activation of Th1-factors could be useful for permitting more plasticity and favouring hybrid phenotypes, or leading to the regulation of STAT1 and STAT4 target genes that are not Th1-specific; it is unclear which form of dimers are formed by STAT1 and STAT4 downstream of IL-4, so the target genes of IL-4-induced pSTAT1 could be different from those of the IFN-γ induced pSTAT1.

The linear regression analysis revealed no direct negative effects of any cytokine on the phosphorylation of any STAT. Although a reduction in pSTAT4 levels could be observed at day 4 of culture in the presence of IL-4, this is most likely explained by the GATA-3-mediated repression of STAT4 after GATA-3 up-regulation downstream of IL-4. Even though suppressors of cytokine signalling (SOCS)[179,180] protein have been implicated in the repression of the IFN-γ[206], IL-12[207] and IL-4[208] pathways, the absence of negative effects on STAT phosphorylation in this study study suggests that SOCS proteins play no cytokine-specific role in primary *in vitro* differentiation, or that their negative effects are generally masked by the STAT-activating cytokine effects. SOCS protein could act by regulating general STAT phosphorylation independently of the cytokine stimuli received by the cells.

5.3 Transcription factor regulation

Master transcription factors such as T-bet and GATA-3 are defined by the fact that they are necessary and sufficient for programming a specific cell fate[69]. This simplified paradigm also considers that a master transcription factor is only expressed in cells having the corresponding cell fate; however, more and more cases of stable co-expression of so-called 'master transcription factors' have been described in differentiated Th cell (e.g., FoxP3 and Bcl-6[209], T-bet and GATA-3[70] or GATA-3 and FoxP3[210]). Furthermore, the discovery of new Th cell lineages relying on STAT signalling[40] and the study of STAT binding and the resulting epigenetic modifications[211] has led to a re-evaluation of the importance and roles of STAT transcription factors for Th cell differentiation. Those facts motivated our detailed analysis of the response of T-bet, GATA-3 and STAT protein dose-response to polarising cytokines.

This study analysed in detail the expression of five transcription factors crucial for Th1 and Th2 cell differentiation: T-bet, GATA-3, STAT1, STAT4 and STAT6. As master transcription factors, T-bet and GATA-3 are responsible for regulating many effector proteins in Th1 and Th2 cells. Although many aspects of their regulation have been studied, we focused on the mechanisms allowing the emergence of mixed Th1-Th2 phenotypes. We discovered that the expression levels of the STAT proteins are also dynamically regulated by the phosphorylated STATs themselves and GATA-3. The regulation of STAT proteins during differentiation partly mediated the rewiring of the signalling pathways, thus modulating the response of the cells to cytokines during a secondary antigen encounter and during primary differentiation, as STAT phosphorylation was found to be long-lasting und could thus be influenced by rewiring during the late phase of differentiation. The effect of TCR-induced transcription factors on T-bet and GATA-3 was also looked into, as high antigen concentrations has been reported to lead to Th1 differentiation and low concentrations to Th2 development[133,134,135,136]. STAT5 downstream of IL-2 co-operate with GATA-3 to induce IL-4 in Th2 cells[108,112,113], but was not found to be differentially expressed in this study (data not shown).

Several other transcription factors that have not been considered here have been implicated in Th1 and Th2 differentiation. Eomes[97], Runx3[98,97], Hlx[99] and Ets transcription factors[100,101] co-operate with T-bet to induce IFN-γ expression, while Onecut2 might form a positive feedback loop on T-bet expression[102]. In the Th2 pathway, c-Maf and junB are involved in IL-4 regulation[212,114,116]. Dec2 is induced by GATA-3 and in turn induces IL-4, IL-5 and IL-3 expression[119,47,123]. T cell factor 1 was shown to up-regulate GATA-3 and repress IFN-γ downstream of TCR signalling[59]. The latter negative regulation was also carried out by c-Maf[114]. Furthermore, Ikaros silences T-bet and IFN-γ in Th2 cells[125,126]. These transcrip-

tion factors would be good candidates for further analysis using dose-response experiments and linear regression; however, the mostly high coefficients of determination obtained with the transcription factor considered here suggest that other factors play a minor role in the determination of the quantitative response during primary differentiation. This observation is in line with the fact that ectopic expression of T-bet or GATA-3 alone, but not of the other transcription factors in isolation, drives the expression of Th1 or Th2 cytokines, respectively.

Antigen concentration does not bias Th cell differentiation when *Il12-/-Ifng-/-* APCs are used

Our data showed no specific correlation between antigen concentration and Th1 or Th2 differentiation, but a general higher protein expression with higher antigen doses. However, a correlation between lineage decision and strength of antigen stimulus has been shown previously[133,134,135,136]. One study[136] attributed the correlation between antigen dose and Th1 differentiation to the up-regulation of CD40L on the Th cells, leading to IL-12 production by the dendritic cells used as APCs; as the APCs used in this study were IL-12-deficient, this effect could not be observed here. Other work[133,134] linked this effect to the abrogation of early IL-2-induced IL-4 production by the Th cells. Another study[135] stated that this bias was unlikely to be due to autocrine IL-4 signalling. However, the mouse strain used (transgenic mice with a TCR specific for the carboxy terminus of pigeon cytochrome c on the B10.a background) was different than the one used in this study (transgenic mice with a TCR specific for the GP_{61-80} petide on the C57BL/6 background), possibly changing the balance of Th1/Th2 cytokines produced upon primary stimulation. Indeed, no other work analysed the response to titrated antigenic peptide in LCMV-specific Th cells. The discrepancy between our data and the published data led us not to consider the dose of antigen triggering TCR signalling any further in this work. A detailed study of the dose-response of signal transcription downstream of the TCR is beyond the frame of this study; it could, however, help infer the mechanisms involved and explain the differences between the experiments shown here and previously published observations.

Phosphorylated STATs play a central role in transcription factor regulation during Th1-Th2 cell differentiation

In general, STAT proteins are known to activate the expression of their target genes, often lineage-defining ones, as is the case with pSTAT1 and pSTAT4 inducing T-bet and pSTAT6 inducing GATA-3; only few direct transcriptional repressive mechanisms have been documented[213,214] until evidence of the induction of repressive epigenetic modifications by the STATs has been published[150]. We found evidence

of several negative regulations by the pSTATs (i.e., the repression of GATA-3 and STAT6 by pSTAT1 and of STAT1, STAT4 and GATA-3 by pSTAT4) and novel positive regulations. Different mechanisms have been proposed for gene regulation by the STATs, including direct initiation of gene regulation and creation of epigenetic patterns around their binding sites[211]. STAT-dependent permissive epigenetic patterns include high levels of H3K4me3, high levels of H3K36me3 and low levels of H3K27me3. In contrast, repressive patterns include high levels of H3K27me3 and low levels of H3K36me3[150]. In agreement with our findings of repressive actions of pSTAT4, STAT4 binding to the *Stat4*, *Gata3* and *Stat1* genes was shown[150]. Although the epigenetic patterns around the binding sites could not be identified as clearly permissive or repressive, the expression of STAT1 and GATA-3 were increased in STAT4-deficient Th1 cells compared to wild-type (by a factor of about 1.5 for STAT1 and 5.9 for GATA-3[150]), consistent with our own observation in differentiating *Stat4-/-* cells. No ChIP-Seq data is available for STAT1 binding in murine Th cells. Information about the binding of STAT1 to the *Gata3* and *Stat6* genes as well as comparisons of their expression levels in differentiating *Stat1-/-* T helper cells would be helpful in testing the predictions of our models on these points. A study of STAT1 DNA-binding activity and induction of epigenetic modifications would be of high relevance, as the central role of IFN-γ-controlled (and also IL-12-controlled) STAT1 in transcription factor expression regulation seems to have been hitherto underappreciated. STAT1 not only acts as an inducer of T-bet, but also auto-activates and potentiates STAT4 signalling as discussed in the previous section and further supports Th1 differentiation by repressing both STAT6 and GATA-3.

An unresolved question is to what extent the intricate activations and inhibitions described here are T cell specific. For example, auto-activation of STAT1 and auto-inhibition of STAT4 might serve specific functions in determining Th cell fate, but might not be relevant in other cell types. T cell specificity might be achieved by enhancers that are selectively active in T cells.

The timescale of T-bet and GATA-3 regulation is shorter than that of STAT regulation

The main assumption we made for the linear regression analysis was that the effects of the pSTATs on transcription factor expression were cumulative over time, and that each transcription factor could have a different 'memory' for binding of pSTATs in the past. This assumption is based on several facts: first, contrary to phosphorylation or de-phosphorylation, which happen in a timescale of minutes to hours[197], protein half-lives are in a timescale of hours to days, meaning that the protein produced following the binding of a transcription factor could still be present in the cell for days once the regulator is not bound anymore. Second,

transcription factors are known to induce epigenetic modifications that modulate the expression level of their targets durably.

During our evaluation of the parameter quantifying memory in the linear models, we found that T-bet and GATA-3 were relatively little influenced by past binding of their regulators in comparison to STAT proteins. These differences could be due to differences in protein half-life (e.g., GATA-3 has a half-life of about 1 hour[215], whereas STAT1's half-life is about 16 to 20 hours[216]) or/and to a different type of regulation: an acute regulation of transcription would have shorter-lasting effects than the induction of epigenetic changes at the target gene's locus. This difference in transcription factor binding 'memory' suggests that GATA-3 and T-bet expression stays plastic during differentiation, and thus can react faster to changes in the signalling environment, whereas STAT expression is more stable. A global mapping of H3K4me3 and H3K27me3 showed that, while cytokine genes show a pattern consistent with terminal commitment in differentiated cell, exhibiting permissive marks for the cells of the corresponding lineage and repressive marks for the others, master transcription factor genes are in a bivalent poised state, i.e., exhibit both repressive and permissive marks[149]. That bivalent state seems to allow a greater expression flexibility[217]. Similarly, the epigenetic patterns on the STAT loci were undetermined; a systematic analysis of the epigenetic changes at the STAT and master transcription factor loci following stimulation with different cytokine doses and combinations could help unravel the mechanisms implicated in the fast versus slow regulation of master transcription factors versus STATs.

We used only one memory parameter per linear model, i.e., the effects of all regulators on a specific target decrease with time at the same rate. This is an assumption we made for simplicity's sake as the models were fitting the data well. However, more accurate models would have a different memory parameter for each regulator, as there is no biological reason suggesting that all transcription factors act in a similar manner on a common target; on the contrary, not only are there repressors and activators, but some transcription factors act on the epigenetic level, other activate or repress transcription more directly while some recruit other transcription factors to the loci they bind to. Thus, a more detailed computational analysis could help further unravel the mechanisms at work.

Synergistic effects of cytokines

In addition to the direct effects of the pSTATs on the expression of T-bet and GATA-3 the linear analysis described, we observed complex effects when visually analysing the data: IFN-γ seemed to potentiate the effects of IL-12; in addition to that, IL-4 diminished the effect of IL-12 on T-bet: in its presence, IL-12 was not sufficient for optimal T-bet expression anymore[82]. Those effects were not directly

described by the equations describing T-bet or GATA-3 expression, neither in the linear models, nor in the dynamical model as only additive effects of the pSTATs were included. However, those observations cannot only be explained by potential direct synergetic effects of the pSTATs on their targets T-bet and GATA-3, but also by indirect effects through the regulation of signalling pathway components seen in this study and other published work. In the case of IFN-γ and IL-12 augmenting each other's effects on T-bet and GATA-3, it could be mediated by T-bet itself: both pSTAT1 and pSTAT4 increase T-bet expression, which in turn up-regulates STAT1 and STAT4. Furthermore, the IL-12Rβ2 chain is up-regulated by IFN-γ and repressed by IL-4[202,122,123], thus modulating the sensitivity of the cells to IL-12. An analogous mechanism could be in place to explain the effect of pSTAT6 on the up-regulation of T-bet: GATA-3 is up-regulated by pSTAT6, and was found to down-regulate both STAT4 and T-bet in this work. This could be why STAT1 activation by IFN-γ is necessary to obtain a unimodal T-bet up-regulation in hybrid Th1/2 cells[82].

STAT regulation showed similar effects of cytokine combinations as master transcription factor expression. The fact that IL-12 does not seem to down-regulate STAT1 in the absence of IFN-γ could be explained by the fact that IFN-γ is needed to up-regulate STAT1, which is expressed only at a basal level in the absence of the latter, and thus cannot be further down-regulated by pSTAT4 downstream of IL-12, or by the fact that IFN-γ is needed to achieve IL-12 responsiveness[96]. The latter could also explain the enhanced repression of STAT4 downstream of IL-12 in the presence of IFN-γ. The reduced effect of IFN-γ on STAT1 in the presence of IL-12 could be linked to the down-regulation of the IFNgRα chain during IL-12-favoured Th1 differentiation[203,204]. The down-regulation of STAT4 in response to IL-4 is, according to our model, GATA-3-mediated and weak compared to other effects on STAT4, and could thus be masked by the up-regulation in response to IFN-γ.

Network rewiring occurs during primary differentiation

We discussed earlier how the kinetics of STAT phosphorylation are controlled, mainly through regulation of total STAT and cytokine receptor expression. These regulations act in a cumulative manner and contribute to the rewiring of the signalling network. Network rewiring is the process that makes the topology of a network dynamic, allowing for sequential effects to lead to a correct final differentiated state. It has been shown to be of importance in several developmental processes[218] and is relevant to cancer biology and treatment[219].

Our data and earlier publication show evidence of extensive network rewiring during Th cell differentiation. One clear example of rewiring in the settings used in this work is the IFN-γ pathway: naive Th cells are IFN-γ responsive, as can

be seen by a fast phosphorylation of STAT1 in presence of IFN-γ. During Th1 differentiation, The IFN-γ receptor is down-regulated[203,204] and no pSTAT1 can be detected in Th1 cells on day 5 of culture, in contrast to pSTAT4 and pSTAT6 that are still present. Similar mechanisms can be observed in other parts of the Th network; indeed, as STAT4 is up-regulated by T-bet and STAT1, so is the IL-12 receptor[96], increasing the potential of IL-12 signalling; the lower levels of IL-4 receptor in Th1 cells are mirrored by the down-regulation of STAT6 by pSTAT1. The effect of the down-regulation of the IFN-γ receptor in Th1 cells[203,204] could be augmented by the repression of STAT1 by pSTAT4. Thus, by regulating the expression of total STAT proteins and cytokine receptors, the potential of the cell to receive a signal is modified, which can help stabilise an acquired phenotype, prevent over-activation of a specific pathway, and regulate the plasticity of the cells[220]. In addition to that, cytokines influence cell proliferation (Supplementary Figure 7.9), which emphasises the need for a fine-tuned control of cytokine responsiveness. Finally, rewiring of the network during differentiation allow the cells to respond differentially to a specific stimulus depending on their environment and could be crucial for permitting graded T-bet and GATA-3 expression. In this context, the rewiring of the network during primary differentiation by long activated STATs could be primordial.

Direct auto-activation of T-bet and GATA-3 does not significantly improve the model's predictions

Auto-activation is an important motif in cell differentiation and lineage decision, as both direct and indirect auto-activating mechanisms of lineage-specific factors can help maintain the cell phenotype. GATA-3 has been shown to trans-activate its own gene[117,118], and while a similar mechanism has been proposed for T-bet, only an indirect loop has been published[102]. Despite these facts, both our linear analysis and dynamical models lack auto-activatory loops. Regulatory loops cannot be described by linear models, so that the topology resulting from our regression analysis could not include them; however, based on the literature, we still tested dynamical models including auto-activatory effects. Surprisingly, the addition of auto-activatory terms for T-bet and GATA-3 did not improve the model fit, suggesting that auto-activation does not play a significant role in the settings under which T-bet and GATA-3 were studied in this work. As we studied the system under external stimulation (i.e., cytokine signals), it could be that those responses are much stronger than auto-activation and thus mask the contribution of the latter. Auto-activation would then become important in the resting cells, once no differentiating cytokine signals are present anymore.

5.4 Cytokine recall responses are predicted well by T-bet and GATA-3 levels

Cytokines are the main effector molecules produced by Th cells and their regulation has been extensively studied. Here, we focused on the response to gradual and mixed Th1-Th2 stimuli, and used the percentage of cytokine producers after TCR re-stimulation in differentiated cells as a quantitative measure of cell function (as cytokine expression is stochastic even in otherwise unimodal T cell populations[221]). Cytokine production can be regulated at the epigenetic level as well as by direct activation of transcription by several transcription factors; here, we summarise some of the regulatory mechanisms relevant for this study modulating the expression of IFN-γ, IL-2, TNF-α, IL-4, IL-13 and IL-10.

The expression of IFN-γ is up-regulated by T-bet[46], STAT4[95,222], Hlx[223], Runx3[98], Eomes[97] and members of the Ets family[100,101]. In contrast, GATA-3 represses IFN-γ expression by inducing repressing histone marks[124,123] and by repressing the Runx3-Eomes-induced up-regulation of IFN-γ expression[97]. However, linear regression analysis found that T-bet alone was a good predictor of the frequency of IFN-γ expressing cells (in agreement with a detailed study of IFN-γ expression in Th1 cells[224]), suggesting that T-bet is the limiting factor among the positive regulators of IFN-γ and that direct negative effects of GATA-3 are negligible in our setup. Alternatively, the per cell amount of IFN-γ, as opposed to the percent of producers, could be regulated by other transcription factors than T-bet, although it was shown that T-bet was also a good predictor of the per-cell amount of IFN-γ[224].

IL-2 production is strongly up-regulated by transcription factors downstream of the TCR such as NFATs, OCT-1 and the NF-κB family members p65 (RelA) and c-Rel[225]. T-bet has been shown to form heterodimers with RelA, thus preventing the up-regulation of IL-2 by the latter[226]; furthermore, Ikaros, a Th2 protein, was shown to repress IL-2 by maintaining hypoacetylated histones[227]. However, linear regression analysis predicted that IL-2 was negatively regulated by GATA-3 and not T-bet, although GATA-3 was not found to bind the IL-2 locus[123]. The effect is unlikely to be mediated by STAT6, as pSTAT6 was not found at the IL-2 locus either[150]. These results suggest that, in this particular setting, IL-2 could be negatively regulated by a GATA-3 controlled factor, or by GATA-3 itself, perhaps via a distal enhancer causing the lack of binding detection in the ChIP-experiments.

TNF-α production is highly regulated in a post-transcriptional manner[228]. The p38-MAPK pathway downstream of LPS positively regulates the stability of TNF-α mRNA[229], while IL-10 negatively interferes with TNF-α production[230]. IFN-γ is linked to TNF-α up-regulation[231]. Absence of IL-4 has been associated with an increase in TNF-α in some settings[232]; furthermore, a decrease in TNF-α was linked with GATA-3 over-expression[233]. Linear regression analysis allowed

to accurately predict TNF-α expression based on GATA-3 expression; while the mechanism underlying this correlation is unclear, the finding is coherent with general knowledge about TNF-α expression being higher in Th1 cells than in Th2 cells.

Although IL-10 has originally been described as a Th2 cytokine[234], it is expressed by Th1 cells in several conditions[235,236]. Several proteins implicated in Th1 and Th2 differentiation have been shown to play a role in IL-10 regulation. The transcription factor c-Maf is essential for IL-10 expression[237]; Lck, a tyrosine kinase, has been associated with the up-regulation of IL-10 in Th1 cells[238]; STAT3 downstream of IL-12-family cytokines has been implicated in positive IL-10 regulation[239]. TGF-β and ICOS are also linked to IL-10 expression[240,241]. As IL-10 expression could not be predicted accurately by T-bet/and or GATA-3, it is probable that its expression is mainly regulated by other transcription factors.

5.5   Limitations of the computational methods

We used linear regression analysis on protein data from multiple stimuli titration experiments to compute a network of Th1 and Th2 signalling. Linear regression is a robust, unbiased method, and allowed us to reconstruct the known signalling network implicated in Th1 and Th2 cell differentiation as well as to discover hitherto undescribed mechanisms. However, it has some limitations, the main one being that this method does not consider causality, which also includes direct versus indirect interactions. Direct interactions can only partially be distinguished from indirect interactions because some factors strongly correlate (e.g., STAT1/STAT4 and T-bet, STAT6 and GATA-3). An experimental approach is necessary to clarify which of two correlating factors is responsible for the observed effect. Linear models describe correlations between factors and contain no information about causality; regulatees and regulators can exchange places without influencing the results of the modelling. Here, we benefitted from being able to assign regulators and regulatees and considered a hierarchy of such regulator-regulatee groups. We used the results of the linear regression analysis as a guide for experiments that established wether STAT effects were direct or mediated by T-bet.

Furthermore, linear regression can only describe signalling going one way, and not discover loops. However, if the active form of a transcription factor can be distinguished from the total protein, as is the case with STAT proteins, auto-activation can be discovered. The linear approach took each time point separately in consideration, thus not explaining any of the dynamics of the networks.

To complement the linear regression approach, we built a dynamical model based on ordinary differential equations. The structure of such a model directly implies causality between factors and makes mechanistic assumptions. Furthermore,

a single model is used to describe all factors at all time points, thus reproducing the dynamics and all interactions of the studied system at once. The model we describe here is able to reproduce the general behaviour of the system and independent data as well as the principal dynamics, thus validating the network topology, there are some remaining discrepancies. Both T-bet and GATA-3 are somewhat over-estimated at late time points; the peak of STAT4 expression present in the data at day 3 is not visible in the model, and STAT6 up-regulation starts too early. The model we used in this work is kept very simple, as each protein is described by a single rate equation for its production. Detailed dynamical models would describe the production of a functional transcription factor through several equations corresponding, for example, to mRNA transcription, mRNA maturation, nuclear export of mRNA, translation to protein, protein modifications and nuclear import of the protein. Thus, our model summarises transcription, translation and eventual post-translational modifications in a single equation, which may partly explain why the kinetics of up-regulation are not reproduced accurately by the model. Furthermore, we postulated that STAT phosphorylation could be described as a linear process depending only on extrapolated cytokine availability. Although the time courses of STAT phosphorylation are reproduced accurately by the model, it should be kept in mind that cytokine concentrations were not measured during the cultures and are thus inferred values based on assumed kinetics for their degradation and consumption. A more detailed and accurate way to model STAT phosphorylation would include receptor availability as well as cytokine concentrations and could be modelled by differential equations.

Another simplification used in this work is the form chosen to model the contribution of each of the transcription factors to the protein production rate. Usually, Hill functions are used to model the probability of a regulator binding the target genes; we decided to simply use the amount of transcription factor. Furthermore, with the exception of the pSTATs cooperating with the antigen signalling to modulate the production of their target, all positive regulatory mechanisms are considered independent, thus, the total production rate is the addition of the basal rate to all positive regulations divided by the sum of all negative regulations. However, synergistic actions of several transcription factors could take place.

5.6 Outlook

In this work, we developed a method for the inference of a gene network downstream of external stimuli and applied it to Th cell differentiation. The method and the insights gained into the analysed network open the way for further research using our approach into different biological systems as well as deeper analysis of the Th1 and Th2 responses to mixed informative cytokine stimuli.

This work left several open questions concerning Th1 and Th1 differentiation under mixed stimuli. Our analysis of STAT phosphorylation suggested that the strength and duration of downstream signalling strongly depended on cytokine and cytokine receptor availability; thus, it would be interesting to combine the approach used here with time-resolved measurements of both cytokine concentration and cytokine receptor expression to gain more insight into the rewiring mechanisms allowing the emergence of hybrid cells and Th cell plasticity. Furthermore, the linear analysis revealed strong correlations between, on the Th1 side, T-bet and pSTAT1 as well as pSTAT4 and, on the Th2 side, GATA-3 and pSTAT6. Although the choice of linear model based on R^2 and AIC values gave clear results as to which, between pSTAT and master transcription factor, was modulating the expression of the target protein, an alternative model substituting one for the other also often gave good results. We clarified this point for the Th1-pathway by using *Tbx21-/-* and *Stat4-/-* cells, thus determining with certainty if the effects observed downstream of IL-12 and IFN-γ were mediated by T-bet or more direct effect of the pSTATs, but the question remains open for GATA-3 and pSTAT6. Thus, it would be informative to perform an IL-4 titration in *Gata3-/-* Th cells to see which of the interactions presented here and GATA-3-independent.

Previous work on hybrid Th1/Th2 cells showed that the hybrid phenotype is stable in the memory phase, i.e., in the absence of external instructive signals, as the Th1 and Th2 phenotypes are. However, the stability of the different expression levels of T-bet and GATA-3 as well as of the components of the signalling pathways in this memory phase has not been studied. In order to determine if resting memory cells still display gradual master transcription factor levels and a gradual response to a secondary activation, cells differentiated with different amounts of cytokines should be transferred into naive recipient mice and their MTF levels quantified in the resting cells. Indeed, even though the dynamical model presented here predicts a continuum of expression levels, it represents the response of the cells to instructive cytokines, and not their memory phenotype in the absence of external cues; such models that include auto-activation and mutual repression of T-bet and GATA-3 typically display only up to four steady states after removal of cytokine signals: a naive state with low T-bet and GATA-3 expression, a Th1 state with high T-bet and low GATA-3, a Th2 state with high GATA-3 and low T-bet and finally a hybrid state with intermediate T-bet and GATA-3 expression. To understand how the cells quantitatively remember their activation history or respond to different amounts and combinations of cytokines during secondary activation would be helpful to comprehend the immune response after vaccination, secondary infections or recurring activation of specific cells, as is the case in allergies and autoimmune diseases.

An interesting point that was not further studied in this work is the 'memory' of transcription factor for the previous binding of their regulators. During the linear regression analysis, we used a parameter λ to quantify how long a target transcription factor was influenced by its regulator. However, we used only one value for each regulated target and applied it to all its regulators, an assumption that is not justified by biological facts, even if it allowed us to find good fitting models. Indeed, as mentioned above, λ summarises several mechanisms: protein half-life is reflected in the λ value, but also long-lasting epigenetic changes at the gene locus as well as modulation of acute transcription. Thus, the use of a different parameter for each regulator could be informative as to the mechanism used by a transcription factor to modulate transcription.

Although we used flow cytometry as a measurement tool, as it is well established in the T cell system, this time-resolved approach to study dose-responses could easily be generalised to other systems and other quantification methods. Indeed, as long as the input signals are well determined and the targets quantifiable (RNA expression by RT-PCR, microarrays or sequencing, protein amounts by flow cytometry, ELISA or western blot), dose-responses can be studied and linear correlations inferred. As such, the method used here could be useful in reconstructing signalling networks from different fields of biology. Detailed prior knowledge about the main network components and the hierarchy between regulatees and regulators as we had is helpful, but not necessary to gain information about the network; general gene annotation would be enough the determine the general hierarchy of a system based on gene function. Thus, the method described in this work could be easily generalised and used at a larger scale; the questions we addressed allowed us to focus on a few key factors and keep the models, both linear and dynamical, simple. Indeed, the dynamical model presented here is a simple one; it could, however, be used as a basis for a more detailed model of Th1 and Th2 differentiation, for example by using more complex functions to model the production rates or by including more factors (e.g., cytokine receptors). Furthermore, such a model could be adapted to include other Th differentiation pathways, like the Th17 or Treg lineages.

5.7 Conclusion

The experimental approach used in this work to study the Th1-Th2 signalling network is straight-forward and focuses mainly on two aspects: the dynamics of up-regulation of the known main players in the network, and the dose-response of these same factors to instructive stimuli. These aspects are studied on multiple levels: signal transduction downstream of instructive stimuli, expression of transcription factors, and finally, expression of effector cytokines as a read-out for cell

function. Basing our study on established knowledge about Th1-Th1 cell differentiation that Th1 versus Th2 cells arise when T-bet versus GATA-3 are up-regulated via IFN-γ/STAT1 and IL-12/STAT4 versus IL-4/STAT6 signalling, leading to Th1- versus Th2 effector cytokine expression, we designed an *in vitro* differentiation protocol allowing us to quantify the main players in Th1-Th2 differentiation in response to the aforementioned cytokines in a time-resolved manner. Specifically, the polarising cytokines IFN-γ, IL-12 and IL-4 were titrated in different combinations, signal transduction was quantified daily by measuring the amounts of phosphorylated STAT1, STAT4 and STAT6 downstream of the cytokines by flow cytometry, and then their target transcription factors T-bet and GATA-3, as well as the total STAT1, STAT4 and STAT6 amounts were likewise quantified daily by flow cytometry. Our functional read-out was the amount of cytokine (i.e., IFN-γ, TNF-α, IL-2, IL-4, IL-13 and IL-10) expressed upon antigen restimulation after differentiation. Linear regression analysis allowed us to infer the regulatory network underlying Th1, Th2 and hybrid Th1/2 cell differentiation in an unbiased manner by selecting the significant relationships between all the analysed factors. This method is potentially applicable to all systems where the dose-responses of known players to external stimuli can be quantified.

Experiments showed that cells receiving distinct cytokine signals during differentiation showed phenotypic characteristics corresponding to all the signals received at the three layers studied: STAT phosphorylation, STAT and master transcription factor expression and signature cytokine expression. The network resulting from this analysis showed that the signals from distinct cytokines were processed independently of each other; indeed, both linear regression analysis and dynamic modelling were able to explain the expression of master transcription factors and STAT proteins by additive effects of the upstream cytokines; although synergistic effects were suggested by the visual analysis, most of them could be explained by independent effect on members of the signalling pathways. Thus, signal integration during differentiation is mediated by network rewiring and not by complex interactions between the transcription factors.

Furthermore, the network predicted several mutually repressive mechanisms between the Th1 and the Th2 pathways: the Th1 master transcription factor T-bet was repressed directly by GATA-3, whereas GATA-3 was down-regulated by pSTAT1 and pSTAT4 downstream of the Th1 cytokines IFN-γ and IL-12. Interactions between pathways was not limited to the expression of master transcription factor, but could also be observed at the level of STAT expression, in the form of STAT1 repressing STAT6 and GATA-3 repressing STAT4, and phosphorylation as IL-4 induce the activation of STAT1 and STAT4 additionally to STAT6. Interestingly, the nature of those mutually repressive interactions led to a continuum of expression levels and not to a digital switch between distinct states.

Finally, we found that the expression of most effector cytokines was governed by T-bet and GATA-3, as their expression levels were good predictors for the fraction of cytokine expressing cells. Thus, cytokine amounts during differentiation determine the amplitude of the functional response upon secondary TCR stimulation, and the presence of a continuum of reachable T-bet-GATA-3 co-expression levels allows for fine-tuning of the type-1 and type-2 immune responses induced by a specific pathogen.

Chapter 6

Materials and methods

6.1 Experimental materials and methods

6.1.1 Differentiation of murine Th cells

Mice

LCMV-TCR^{tg} (SMARTA1)[242] mice expressing a TCR specific for the LCMV epitope GP$_{61-80}$ on C57BL/6 background were used as organ donors for the isolation of splenocytes and lymph node cells to obtain Th cells. When indicated, TCR^{tg} mice were crossed with *Ifng-/-* mice[243], *Il4-/-* mice[244], *Tbx21-/-* mice[245] or *Stat4-/-* mice[246]. *Il12p40-/-*[247] x *Ifng-/-* mice with a wild-type TCR were used as organ donors for the isolation of splenocytes to obtain APCs. All mice were all on C57BL/6 background. Mice were bred under specific pathogen-free conditions at the Charité, Berlin or at the Federal Institute for Risk Assessment (BfR), Berlin. All animal experiments were performed in accordance with the German law for animal protection with permission from the local veterinary offices.

Isolation of naive Th cells

Spleens and lymph nodes of 6-8 weeks old SMARTA1 mice were mechanically disrupted to obtain single-cell suspensions. Erythrocytes were lysed by a 3-minutes incubation in erythrocyte lysis buffer (10 mM KHCO3, 155 mM NH4Cl, 0.1 mM EDTA, pH 7.5). Naive Th cells were then enriched by depleting CD8+ cells, macrophages, B cells, NK cells, dendritic cells, granulocytes, Treg cells and acute effector Th cells using magnetic-activated cell sorting; cells were first incubated for 10 minutes on ice with biotin-conjugated antibodies (BD biosciences) specific to CD8a (53-6.7), CD11b (M1/70), CD19 (1D3),NK1.1 (PK136) CD11c (HL3), Gr-1 (RB6-8C5), CD25 (7D4) and CXCR3 (CXCR3-173), respectively, then with anti-biotin microbeads (Miltenyi Biotec) for 10 minutes at 4°C. Cells were then separated

using LS columns (Miltenyi Biotec). Enriched naive CD4+ T cells were stained with PE-Cy7-conjugated anti-CD4 (RM4-5), Pacific Blue-conjugated anti-CD44 PE-Cy7, FITC-conjugated anti-CD62L (MEL-14) and PerCP-conjugated Streptavidin (all from BD biosciences) before Streptavidin-negative CD4+CD62LhiCD44lowere sorted using a FACS Aria II (Becton Dickinson) to a purity >99%.

Isolation of APCs

Spleens and mesenteric lymph nodes of *Il12p40-/-*[247] x *Ifng-/-* mice were mechanically disrupted to obtain single-cell suspensions. Erythrocytes were lysed by a 3-minutes incubation in erythrocyte lysis buffer (10 mM KHCO3, 155 mM NH4Cl, 0.1 mM EDTA, pH 7.5). T cells were then sorted out from the APC fraction using LS columns or an autoMACS separator (both from Miltenyi Biotec) after a 10-minutes incubation on ice with biotin-conjugated anti-Thy1.2 (53-2.1, BD biosciences) followed by a 10-minutes incubation at 4°C with anti-biotin microbeads (Miltenyi Biotec).

T Cell Activation and Differentiation

Naive CD4+CD62LhiCD44loCD25-CXCR3- Th cells were cultured in RPMI 1640 +GlutaMax-I supplemented with 10% (v/v) FCS (Gibco), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), and β-mercaptoethanol (50 ng/ml; Sigma). Cultures were prepared in the presence of *Il12p40-/-xIfng-/-* or wild-type APCs, 0.5 mg/ml LCMV-GP$_{61-80}$, (R. Volkmer, Institute for Med. Immunology, Charité) and 5 ng/ml IL-2 (R&D Systems).

For Th1 differentiation, 10 ng/ml IFN-γ, 5 ng/ml IL-12 (R&D Systems) and 10 μg/ml anti-IL-4 (11B11) were added, unless specified otherwise. For Th2 differentiation, 10 ng/ml IL-4 (R&D Systems) and 10 μg/ml anti-IFN-γ (AN18.17.24) were added. Hybrid Th1/2 cells were cultured with 10 ng/ml IFN-γ, 5 ng/ml IL-12 and 10 ng/ml IL-4. For some experiments, cells were cultured under neutral conditions with 10 μg anti-IL-12 (C17.8), 10 μg anti-IFN-γ and 10 μg anti-IL-4. Cell cultures were split on d2 or d3 and analysed until d5.

6.1.2 Flow Cytometry

Samples were acquired on a FACS Canto II (Becton Dickinson) and analysed with FlowJo (TreeStar). Dead cells and doublets were excluded by a combination of forward scatter height and width gating and a LIVE/DEAD fixable dye (Invitrogen). If not specified otherwise, cells were stained for 15 minutes at 4°C in a volume of 50 μ in the presence of 10 μg/ml anti-FcγRII/III (2.4G2, ATCC) and 2.5 μg/ml purified Rat IgG (Jackson Immunoresearch) and washed with an excess volume.

Intracellular cytokine staining

For intracellular analysis of cytokines, cells were restimulated on d5 with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 4 h with addition of brefeldin A (5 mg/ml; all from Sigma-Aldrich) after 30 min. Following restimulation, cells were stained with a LIVE/DEAD fixable dye (Invitrogen) and fixed in 2% formaldehyde (Merck). Intracellular staining was performed in PBS/0.2% BSA containing 0.05% saponin (Sigma-Aldrich) for permeabilization. Samples were stained with antibodies (eBioscience) specific to CD4 (GK1.5), IFN-γ (XMG1.2), IL-4 (11B11), IL-10 (JES5-16E3), IL-13 (38213.11, eBio13A), TNF-a (MP6-XT22) and IL-2 (JES6-5H4).

STAT staining

STAT protein amounts and phosphorylation of STAT proteins were analysed using BD Phosflow buffers according to the manufacturer's instructions (BD Bioscience). Cells were fixed with prewarmed 1x BD Phosflow Lyse/Fix Buffer for 10 min at 37°C. Cells were permeabilized with ice-cold BD Phosflow Perm Buffer III for 30 min on ice. Then, cells were stained for 30 min with anti-CD4 and either PE-conjugated anti-pSTAT1 (4a) and Alexa-647-conjugated anti-STAT1 (1/Stat1) or PE-conjugated anti-pSTAT6 (pY641) and Alexa-647-conjugated anti-STAT6 (23/STAT6) or PE-conjugated anti-pSTAT4 (38/p-Stat4; all from BD Biosciences) and polyclonal rabbit anti-STAT4 (Zymed). Secondary antibody (Cy5-conjugated donkey anti-rabbit; Jackson Immunoresearch) was added at a final concentration of 0.2 mg/ml. Cells were washed and analysed by FACS. Geometric mean indices were calculated by dividing the geometric mean of the analysed population by the geometric mean of the respective isotype control-stained cells.

T-bet and GATA-3 staining

T-bet and GATA-3 protein amounts were analysed using FoxP3 staining buffer set (eBioscience) according to the manufacturer's instructions. Briefly, cells were stained with anti-CD4 (GK1.5) and a LIVE/DEAD fixable dye (Invitrogen), followed by fixation with 1x Fixation/Permeabilization buffer and intracellular staining with Pacific Blue-conjugated anti-T-bet (4B10) and Alexa-647-conjugated anti-GATA-3 (TWAJ, both from eBioscience) in 1x Permeabilization buffer. Cells were washed in 1x Permeabilization buffer and analysed by FACS. For T-bet/pSTAT4 and GATA-3/pSTAT6 co-stainings, the STAT staining protocol was used. Geometric mean indices were calculated by dividing the geometric mean of stained cells by the geometric mean of the respective isotype control-stained cells.

6.1.3 RNA

mRNA from Th cell differentiation kinetics was purified using the NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturers instructions. Illumina mouse WG-6 gene arrays were prepared, and gene expression was analysed. Differential gene expression was analysed using the EDGE software package[248].

6.2 Computational methods

6.2.1 Data processing

The data was obtained by flow cytometry using the DIVA software (BD biosciences), and the geometric mean of each population was computed with FlowJo (Treestar) after appropriate gating. It was then processed the following way as described below.

Cytokine concentrations

The concentrations $[\mathrm{C}]_{\mathrm{cyto}}$ of IFN-γ, IL-12 and IL-4 used for each condition i were transformed the following way before fitting the linear regression models to the relative pSTAT values:

$$\mathrm{C}_{\mathrm{cyto}}^{i} = \begin{cases} \log_{10}\left(\frac{([\mathrm{C}]_{\mathrm{cyto}}^{i+1})^2}{[\mathrm{C}]_{\mathrm{cyto}}^{i+2}}\right) & \text{if } [\mathrm{C}]_{\mathrm{cyto}}^{i} = 0, \\ \log_{10}([\mathrm{C}]_{\mathrm{cyto}}^{i}) & \text{else.} \end{cases}$$

This transformation approximates the 0 concentration by the next smallest titration step. Furthermore, the $[\mathrm{C}]_{\mathrm{cyto}}^{i}$ were normalised to be between 0 and 1 by dividing them by their minimal value before the log transformation, and again by their maximal value after it.

Transcription factors

For each transcription factor (TF, i.e., T-bet. GATA-3, STAT1, STAT4, STAT6, pSTAT1, pSTAT4 and pSTAT6) staining of condition i, the geometric mean (GM) of the population was divided by the geometric mean of the corresponding isotope control to correct for differences in cell size and autofluorescence, obtaining the geometric mean index (GMI):

$$\mathrm{GMI}_{\mathrm{TF}}^{i} = \frac{\mathrm{GM}_{\mathrm{TF}}^{i}}{\mathrm{GM}_{\mathrm{iso}}^{i}}$$

For each experiment e and transcription factor, this GMI value was further divided by the maximal GMI value for the same TF in the experiment x to correct for

differences in staining intensities and cytometer sensibility between independent experiments:

$$\text{GMI}^i_{\text{TF}, e} = \frac{\text{GMI}^i_{\text{TF}, e}}{\max(\text{GMI}_{\text{TF}, e})}$$

For the analysis of the cytokine effects on the STAT phosphorylation, we computed the relative GMI (rGMI) of each pSTAT (pSTAT1, pSTAT4 and pSTAT6) by dividing the GMI of the pSTAT staining by the GMI of the corresponding total STAT staining after removing the baseline of pSTAT expression:

$$\text{rGMI}^i_{\text{pSTAT}, x} = \frac{\text{GMI}^i_{\text{pSTAT}, x} - \min(\text{GMI}_{\text{pSTAT}, x})}{\text{GMI}^i_{\text{STAT}, x}}$$

Furthermore, in order to be able to compare the weights of the regressors of the linear models, the data for each protein used as an explanatory variable in the linear modelling was further normalised to have a mean of 0 and a standard deviation of 1:

$$\text{GMI}^i_{\text{TF}} = \frac{\text{GMI}^i_{\text{TF}} - \text{mean}(\text{GMI}_{\text{TF}})}{\text{stdev}(\text{GMI}_{\text{TF}})}$$

6.2.2 Linear regression models

The linear modelling was done with Wolfram Mathematica 10. The *LinearModelFit* function was used to fit the parameters to the data as well as to compute R^2, Akaike's Information Criterion (AIC), the statistical significance of the parameters, their confidence intervals, the residuals and Cook's distance. Unless specified otherwise, the models were fitted to the data generated by the IFN-γ, IL-12 and IL-4 titrations performed in the presence and absence of the other cytokines with *Ifng-/-* (IFN-γ and IL-12 titrations) or *Il4-/-* (IL-4 titrations) cells.

The linear regression models were compared using several statistics. The coefficient of determination

$$R^2 = 1 - \frac{\text{SS}_t}{\text{SS}_r}$$

where SS_t is the total sum of squares and SS_r the residual sum of squares, informs about the goodness of fit. The Akaike information criterion, in the case of linear regression models,

$$\text{AIC} = 2k + n \ln(\text{SS}_r)$$

where k is the number of parameters and n the sample size penalises the complexity of the model while rewarding goodness of fit, and the significance of the parameters according to the F-statistic in ANOVA calculations[182].

pSTATs as functions of cytokine concentrations

The family of linear models explaining the pSTAT values in condition i as functions of the cytokine concentrations was build as follows:

$$\text{rGMI}_x^i = \alpha_{x,0} + \sum_{j=1}^{3} \alpha_{x,j} \cdot \text{C}_j^i$$

where $x = \{\text{pSTAT1, pSTAT4, pSTAT6}\}$ and $j = \{\text{IFN-}\gamma\text{, IL-12, IL-4}\}$. For each pSTAT on each day, seven models with different explanatory variables were fitted to the relative pSTAT values: IFN-γ only, IL-12 only, IL-4 only, IFN-γ and IL-12, IFN-γ and IL-4, IL-12 and IL-4 or all three cytokines were used as explanatory variables. The models were compared using the R^2, the AIC and the significance of the parameters. The best model was the one having the lowest AICs and only significant parameters.

Master transcription factors as functions of pSTATs and each other

The family of linear models explaining the expression of the transcription factors T-bet and GATA-3 was built similarly to the previous family, but weighted sums over time until day T of the active transcription factors (pSTAT1, pSTAT4, pSTAT6 and GATA-3 or T-bet, respectively) were used as explanatory variables for T-bet and GATA-3 in each condition i on day T. The transcription factor modelled could not be included in the explanatory variables due to the nature of linear regression analysis.

$$\text{GMI}_x^i(T) = \alpha_{x,0} + \sum_{j \neq i, j=1}^{5} \left(\alpha_{x,j} \cdot \sum_{t=1}^{T} e^{-\lambda_x \cdot (T-t)} \text{GMI}_j^i(t) \right)$$

where $x = \{\text{T-bet, GATA-3}\}$, $j = \begin{cases} \{\text{GATA-3, pSTAT1, pSTAT4, pSTAT6}\} & x = \text{T-bet}, \\ \{\text{T-bet, pSTAT1, pSTAT4, pSTAT6}\} & x = \text{GATA-3} \end{cases}$

and $T = \{1, 2, 3, 4, 5\}$. Similarly to the method used with the models explaining STAT phosphorylation, fifteen models with all possible combinations of the four explanatory variables were fitted to the GMIs of T-bet and GATA-3 independently for each day. λ was determined first by comparing the R^2 of the fitted model including all explanatory variables for increasing λ values and choosing the λ value leading to the highest R2. The models with fixed λ values were then fitted again and compared using the AIC, R^2 and the significance of the parameters. The best model was the one having the lowest AIC and only significant parameters.

STATs as functions of activated transcription factors

The family of linear models explaining the expression of the three STAT transcription factors was build like the one explaining T-bet and GATA-3 expression, using the sums over time until day T of the active transcription factors (T-bet, GATA-3, pSTAT1, pSTAT4, pSTAT6) as explanatory variables for total STAT expression on day T in each condition i.

$$\text{GMI}_x^i(T) = \alpha_{x,0} + \sum_{j=1}^{5} \left(\alpha_{x,j} \cdot \sum_{t=1}^{T} e^{-\lambda_x \cdot (T-t)} \text{GMI}_j^i(t) \right)$$

where $T = \{1, 2, 3, 4, 5\}$, $x = \{$STAT1, STAT4, STAT6$\}$ and $j = \{$T-bet, GATA-3, pSTAT1, pSTAT4, pSTAT6$\}$. Again, thirty-one models with all possible combinations of the five explanatory variables were fitted to the GMIs of STAT1, STAT4 or STAT6 independently for each day. λ was determined first by comparing the R^2 of the fitted model including all explanatory variables for increasing λ values and choosing the λ value leading to the highest R2. The models with fixed λ values were then fitted again and compared using the AIC, R^2 and the significance of the parameters. The best model was the one having the lowest AIC and only significant parameters.

Cytokine producers as functions of T-bet and GATA-3

The percentage of cytokine producers during the recall response after five days of differentiation was expressed as a function of the T-bet and GATA-3 GMIs on day 5 before TCR restimulation in each condition i. Linear functions were fitted to the flow cytometry data of cytokine expression:

$$P_x^i = \alpha_{x,0} + \sum_{j=1}^{2} \alpha_{x,i} \cdot \text{GMI}_j^i$$

where P is the percentage of cytokine producers, $x = \{$IFN-γ, IL-13, IL-4, TNF-α, IL-2, IL-10$\}$, and $j = \{$T-bet, GATA-3$\}$. Three different models were fitted for each cytokine to the data of the IL-12/IL-4 cross-titration performed in wild-type cells, taking both T-bet and GATA-3, only T-bet or only GATA-3 as explanatory variables. The models were compared using the AIC, R^2 and the significance of the parameters. The best model was the one having the lowest AIC and only significant parameters. These linear models were used as a basis to empirically find better-fitting non-linear models using the *NonLinearModelFit* function.

The standardized residuals were examined for each model, as well as Cook's distance, both provided by the *LinearModelFit* function. Three data points were

excluded after analysis of Cook's distance because of biologically aberrant STAT4 or STAT1 GMI due to abnormally low isotope control stainings.

6.2.3   ODE model

Based on the network topology derived from the linear models, a system of ordinary differential equations was designed to describe the data. This system was implemented in MATLAB (MathWorks) using the Data2Dynamics environment[185,186].

Model development

The model used describes the phosphorylation of the STATs in response to polarising cytokines as well as the expression of transcriptions factors in function of active transcription factors. The first part of the model is not ODE-based and describes the kinetics of cytokine concentrations and the phosphorylation of the STATs:

$$[\text{IFN-}\gamma] = C_{\text{IFN-}\gamma} \cdot (1 - \tanh(t - T_{\text{IFN-}\gamma}))$$

$$[\text{IL-12}] = C_{\text{IL-12}} \cdot (1 - \tanh(t - T_{\text{IL-12}}))$$

$$[\text{IL-4}] = C_{\text{IL-4}} \cdot (1 - \tanh(t - T_{\text{IL-4}}))$$

$$\text{pS1} = (\alpha_1[\text{IFN-}\gamma] + \alpha_2[\text{IL-12}] + \alpha_3[\text{IL-4}]) \cdot \text{S1}$$

$$\text{pS4} = (\alpha_4[\text{IL-12}] + \alpha_5[\text{IL-4}]) \cdot \text{S4}$$

$$\text{pS6} = (\alpha_5[\text{IL-4}]) \cdot \text{S6}$$

Where Cs are the logarithm of the cytokine concentrations, t the time in days, the Ts the half-lives of the cytokine concentrations, and the αs the strength of the cytokine effects on STAT phosphorylation. The T and α parameters were fitted to the pSTAT intensities from the IFN-γ, IL-12 and IL-4 titrations, taking the experimental values of the total STAT intensities as inputs.

The transcription factor expression was modeled by a system of five ordinary differential equations, one for each protein. The pSTATs were assumed to act in concert with antigen signalling, so that a linear equation describing the antigen

stimulus was added to the system:

$$\mathrm{Ag} = (1 - e^{(-t)}) \cdot e^{(-l_2 \cdot t)}$$

$$\dot{\mathrm{S1}} = \frac{B_{\mathrm{S1}} + \beta_1 \cdot \mathrm{T} + \beta_2 \cdot \mathrm{pS1} \cdot \mathrm{Ag}}{1 + \beta_3 \cdot \mathrm{pS4} \cdot \mathrm{Ag}} - \delta_{\mathrm{S1}} \cdot \mathrm{S1}$$

$$\dot{\mathrm{S4}} = \frac{B_{\mathrm{S4}} + \beta_4 \cdot \mathrm{T}}{1 + \beta_5 \cdot \mathrm{pS4} \cdot \mathrm{Ag} + \beta_6 \cdot \mathrm{G}} - \delta_{\mathrm{S4}} \cdot \mathrm{S4}$$

$$\dot{\mathrm{S6}} = \frac{B_{\mathrm{S6}} + \beta_7 \cdot \mathrm{pS6} \cdot \mathrm{Ag}}{1 + \beta_8 \cdot \mathrm{pS1} \cdot \mathrm{Ag}} - \delta_{\mathrm{S6}} \cdot \mathrm{S6}$$

$$\dot{\mathrm{T}} = \frac{B_{\mathrm{T}} + \beta_9 \cdot \mathrm{pS1} \cdot \mathrm{Ag} + \beta_{10} \cdot \mathrm{pS4} \cdot \mathrm{Ag}}{1 + \beta_{11} \cdot \mathrm{G}} - \delta_{\mathrm{T}} \cdot \mathrm{T}$$

$$\dot{\mathrm{G}} = \frac{B_{\mathrm{G}} + \beta_{12} \cdot \mathrm{pS6} \cdot \mathrm{Ag}}{1 + \beta_{13} \cdot \mathrm{pS1} \cdot \mathrm{Ag} + \beta_{14} \cdot \mathrm{pS4} \cdot \mathrm{Ag}} - \delta_{\mathrm{G}} \cdot \mathrm{G}$$

S1, S4, S6, T and G represent STAT1, STAT4, STAT6, T-bet and GATA-3 protein expression, respectively. Ag represents the antigen stimulus, which increases with time constant 1 and decreases with time constant l_2 (Figure 4.2). pS1, pS4 and pS6 represent the phosphorylated forms of SATA1, STAT4 and STAT6, respectively. Each protein is produced with a basal rate B_i and degraded with a rate δ_i. In addition to that, the positive and negative regulatory mechanisms described by the linear regression analysis are added to the production rate with weights β_i.

This model describes the positive effects of pSTAT1 on STAT1 and T-bet expression as well as its negative effects on GATA-3 and STAT6, the negative effects of pSTAT4 on STAT1, STAT4 and GATA-3 and its positive effect on T-bet, the positive effects of pSTAT6 on itself and GATA-3. Described are also the positive effects of T-bet on STAT1 and STAT4 and the negative effects of GATA-3 on STAT4 and T-bet. All 19 β parameters were fitted to the whole data of T-bet, GATA-3, STAT1, STAT4 and STAT6 expression from day to day 5 using only the cytokine concentrations as input data.

Model fitting and testing

The model was fitted in two steps; first, the algebraic part of the model describing STAT phosphorylation was fitted to the pSTAT data with the initial cytokine concentrations and STAT values as inputs; then, using the best-fit parameters from the first step, the non-linear part was fitted to the whole dataset using only the initial cytokine concentrations as inputs.

The first part of the model was fitted with Wolfram Mathematica 10. The *NonLinearModelFit* function was used to fit the parameters to the data as well as to compute their confidence intervals. The ODE model was fitted and analysed

using the Data2Dynamics software[185,186]. The parameter estimation procedure was done with the *lsqnonlin* algorithm, a deterministic optimisation algorithm. The confidence intervals and identifiability of the parameters were inferred by calculating the profile likelihood[188,189]. Latin Hypercube Sampling[187] of the initial parameter values was used to verify that a global minimum was found. Alternative models lacking a specific interaction were similarly fitted to the same data.

The accuracy of the model was tested by predicting the T-bet and GATA-3 values from the IL-12/IL-4 cross-titration experiment (which were not used for the fit) consisting of 49 conditions on day 5 of culture using MATLAB and the best-fit parameters. As only endogenous IFN-γ was present during this culture, the concentration of this cytokine was approximated using the percent of producers during the recall response, postulating that it was proportional to the production during differentiation and that the highest production was sufficient to reach saturation (i.e., $C^i_{\text{IFN-}\gamma} = \frac{P^i_{\text{IFN-}\gamma}}{\max(P_{\text{IFN-}\gamma})}$). Using the values for the β parameters found by fitting to the other dataset, the known IL-12 and IL-4 concentrations and the extrapolated IFN-γ concentrations, the model was able to reproduce the levels of T-bet and GATA-3 expression from the experimental data.

Different models were compared using the Akaike information criterion

$$\text{AIC} = 2k - 2\ln\left(\hat{\mathcal{L}}\right)$$

where $\hat{\mathcal{L}}$ is the maximum likelihood estimator computed with the D2D framework.

List of Figures

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Chapter 7

Appendix

7.1 Supplementary figures

IFN-γ titrations

Condition	IFN-γ ng/ml	IL-12 ng/ml	IL-4 ng/ml	cell type
TCR only	0	0	0	Ifng KO
1 IFN-γ	0	0	anti-IL-4	Ifng KO
	0,003	0	anti-IL-4	Ifng KO
	0,03	0	anti-IL-4	Ifng KO
	0,3	0	anti-IL-4	Ifng KO
	3	0	anti-IL-4	Ifng KO
2 IFN-γ + IL-12	0	5	anti-IL-4	Ifng KO
	0,003	5	anti-IL-4	Ifng KO
	0,03	5	anti-IL-4	Ifng KO
	0,3	5	anti-IL-4	Ifng KO
	3	5	anti-IL-4	Ifng KO
3 IFN-γ + IL-4	0	0	10	Ifng KO
	0,003	0	10	Ifng KO
	0,03	0	10	Ifng KO
	0,3	0	10	Ifng KO
	3	0	10	Ifng KO
4 IFN-γ + IL-4 + IL-12	0	5	10	Ifng KO
	0,003	5	10	Ifng KO
	0,03	5	10	Ifng KO
	0,3	5	10	Ifng KO
	3	5	10	Ifng KO

IL-4 titrations

Condition	IFN-γ ng/ml	IL-12 ng/ml	IL-4 ng/ml	cell type
TCR only	0	0	0	Il4 KO
Th1	10	5	anti-IL-4	Il4 KO
Th0	anti-IFN-γ	0	anti-IL-4	Il4 KO
9 IL-4	anti-IFN-γ	0	0	Il4 KO
	anti-IFN-γ	0	0,5	Il4 KO
	anti-IFN-γ	0	5	Il4 KO
	anti-IFN-γ	0	50	Il4 KO
10 IL-4 + IFN-γ	10	0	0	Il4 KO
	10	0	0,5	Il4 KO
	10	0	5	Il4 KO
	10	0	50	Il4 KO
11 IL-4 + IL-12	anti-IFN-γ	5	0	Il4 KO
	anti-IFN-γ	5	0,5	Il4 KO
	anti-IFN-γ	5	5	Il4 KO
	anti-IFN-γ	5	50	Il4 KO
12 IL-4 + IL-12 + IFN-γ	10	5	0	Il4 KO
	10	5	0,5	Il4 KO
	10	5	5	Il4 KO
	10	5	50	Il4 KO

IL-12 titrations

Condition	IFN-γ ng/ml	IL-12 ng/ml	IL-4 ng/ml	cell type
TCR only	0	0	0	Ifng KO
Th2	anti-IFN-γ	anti-IL-12	10	Ifng KO
5 IL-12	0	0	anti-IL-4	Ifng KO
	0	0,05	anti-IL-4	Ifng KO
	0	1,5	anti-IL-4	Ifng KO
	0	45	anti-IL-4	Ifng KO
6 IL-12 + IFN-γ	10	0	anti-IL-4	Ifng KO
	10	0,05	anti-IL-4	Ifng KO
	10	1,5	anti-IL-4	Ifng KO
	10	45	anti-IL-4	Ifng KO
7 IL-12 + IL-4	0	0	10	Ifng KO
	0	0,05	10	Ifng KO
	0	1,5	10	Ifng KO
	0	45	10	Ifng KO
8 IL-12 + IL-4 + IFN-γ	10	0	10	Ifng KO
	10	0,05	10	Ifng KO
	10	1,5	10	Ifng KO
	10	45	10	Ifng KO

Figure 7.1: List of all the different culture conditions used during primary differentiation in the main titration experiments. The cytokines IFN-γ, IL-12 and IL-4 were titrated during differentiation of appropriate cytokine-deficient naive Th cells in presence or absence of one or both other cytokines.
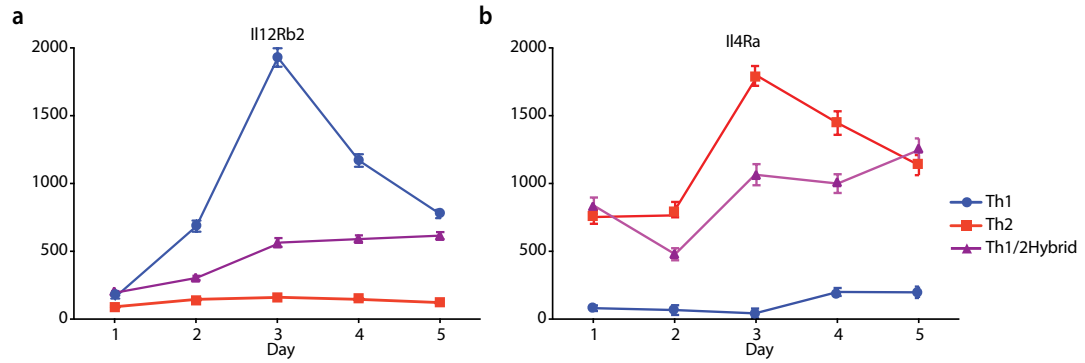
Figure 7.2: Kinetic RNA data of cytokine receptors during differentiation of naive wild-type Th cells. RNA from wild-type Th1, Th2 and Hybrid Th1/2 cells was extracted and quantified by microarrays in a kinetic manner during primary differentiation.
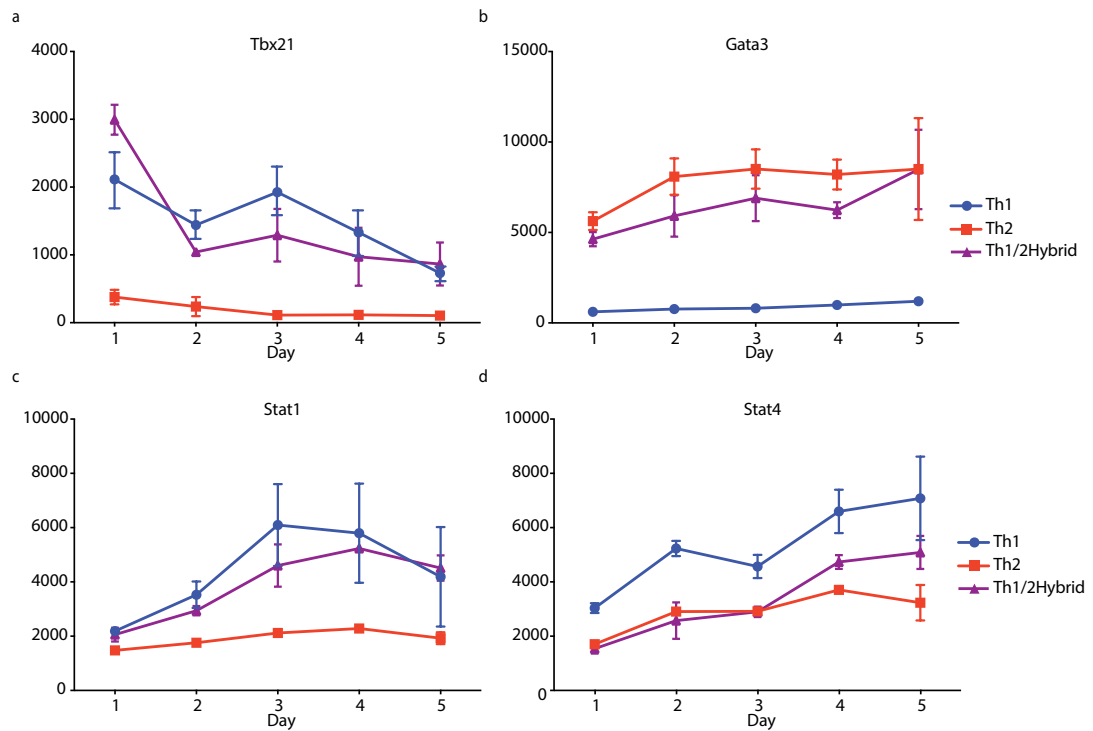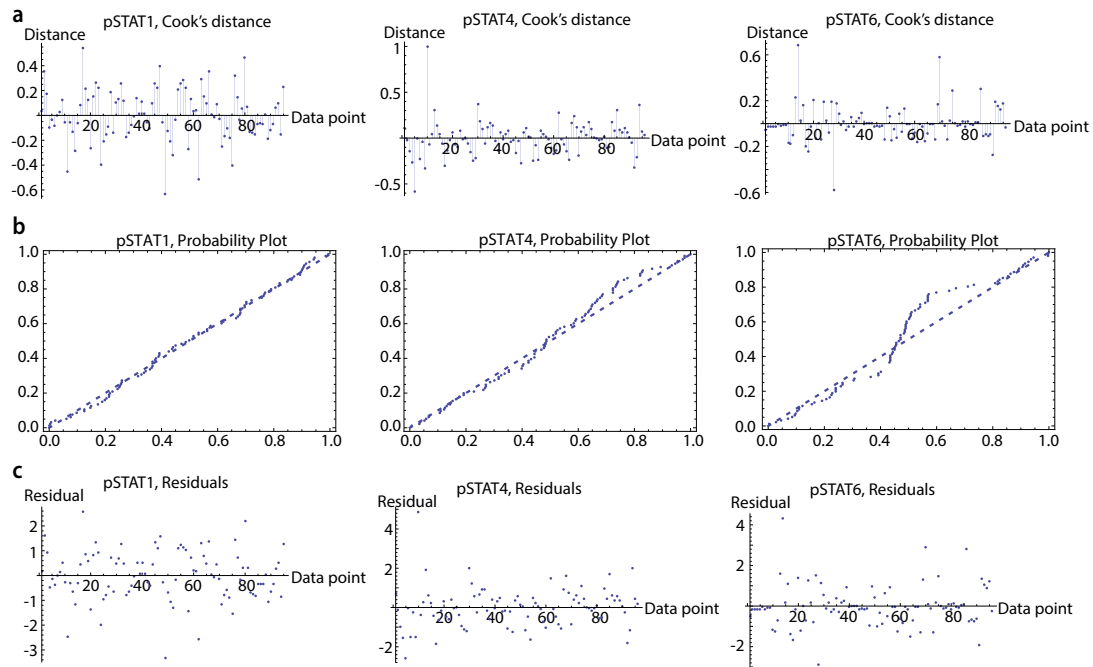

Figure 7.3: Kinetic RNA data of transcription factors during differentiation of naive wild-type Th cells. RNA from wild-type Th1, Th2 and Hybrid Th1/2 cells was extracted and quantified by microarrays in a kinetic manner during primary differentiation.

Figure 7.4: Diagnostics of the linear models for STAT phosphorylation. a Cook's distance for each data point. **b** Normal probability plots. **c** Residual for each data point.
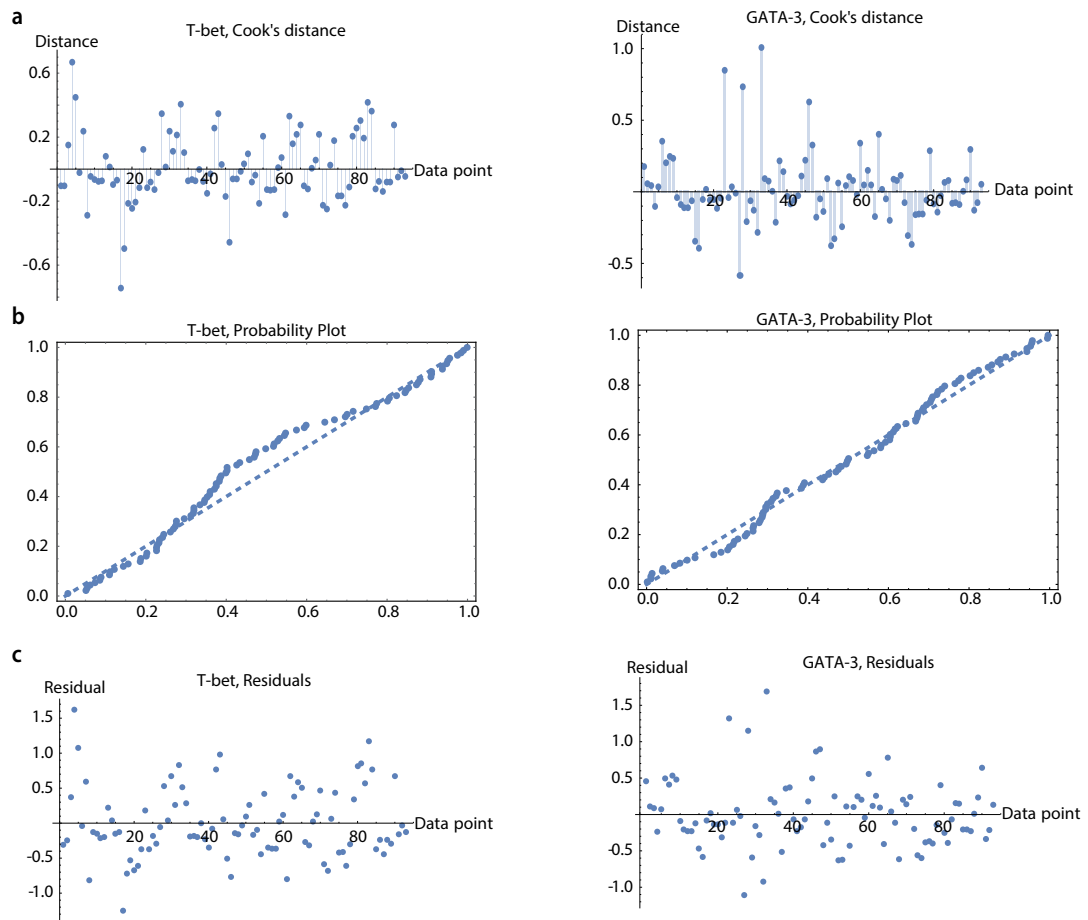
Figure 7.5: Diagnostics of the linear models for master transcription factor expression. a Cook's distance for each data point. **b** Normal probability plots. **c** Residual for each data point.
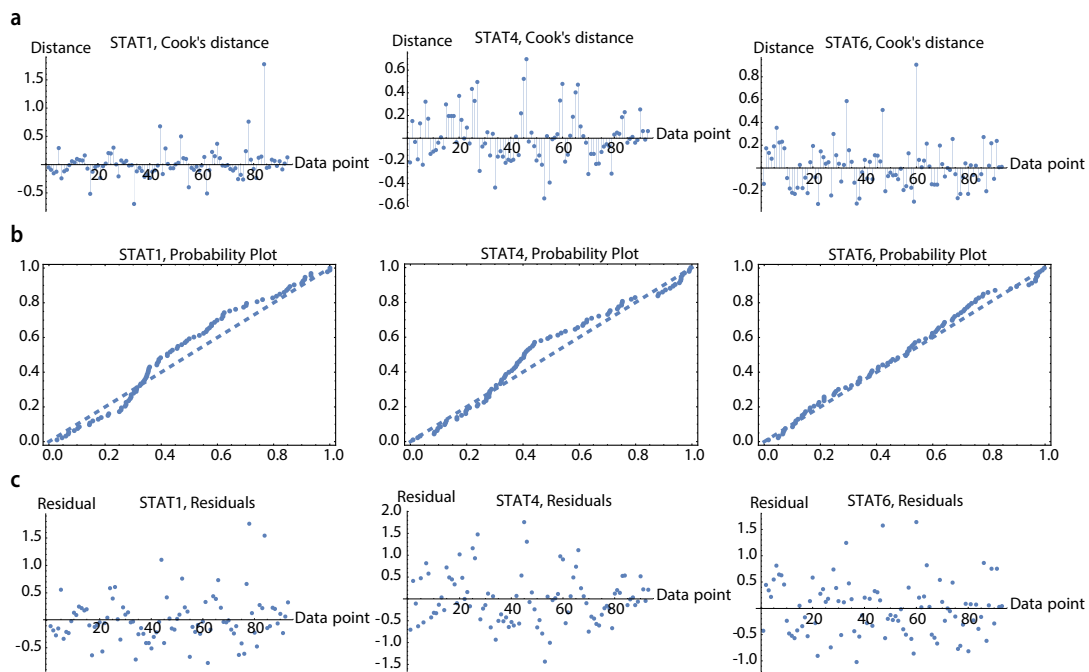
Figure 7.6: Diagnostics of the linear models for STAT expression. a Cook's distance for each data point.
b Normal probability plots. **c** Residual for each data point.
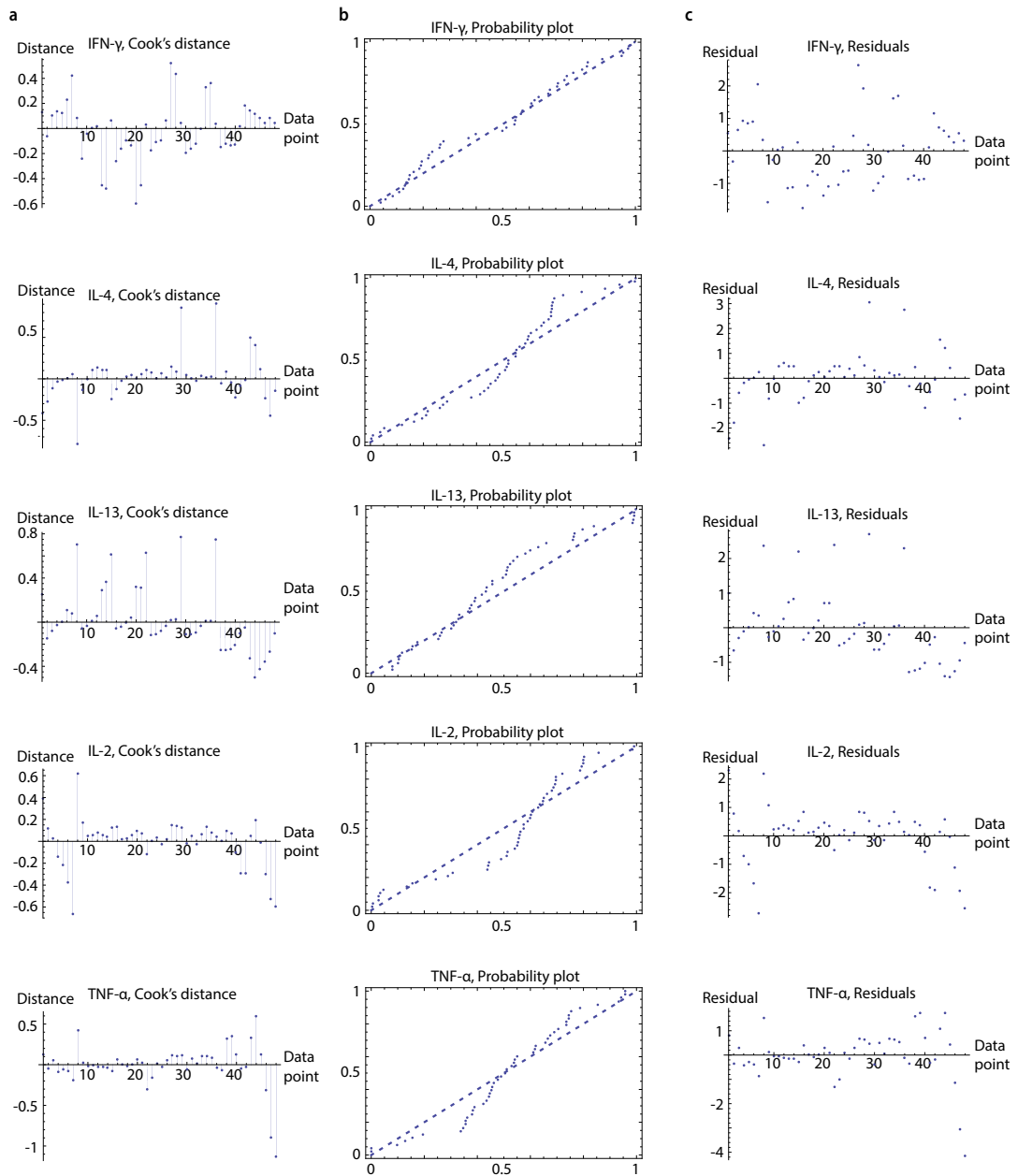
Figure 7.7: Diagnostics of the linear models for cytokine expression. a Cook's distance for each data point. **b** Normal probability plots. **c** Residual for each data point.
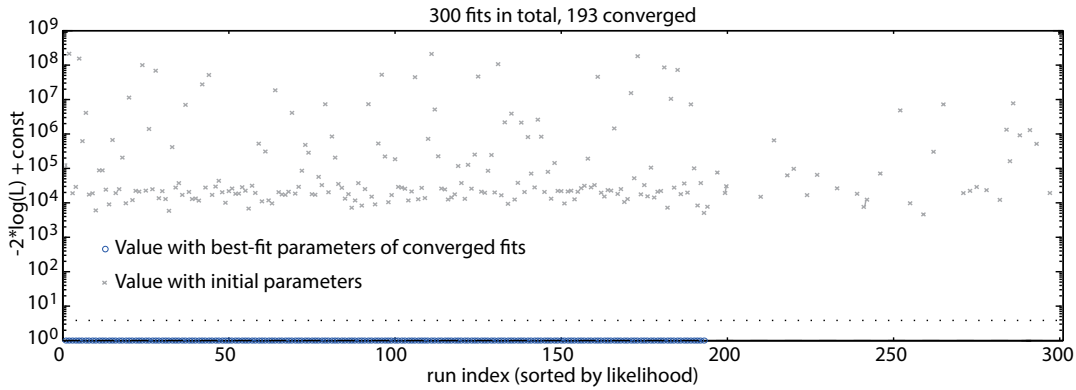
Figure 7.8: Fits of the dynamical models converged to a global minimum. The dynamical model was fitted 300 times with different initial values determined by Latin Hypercube Sampling and the resulting logarithms of the likelihood values compared.
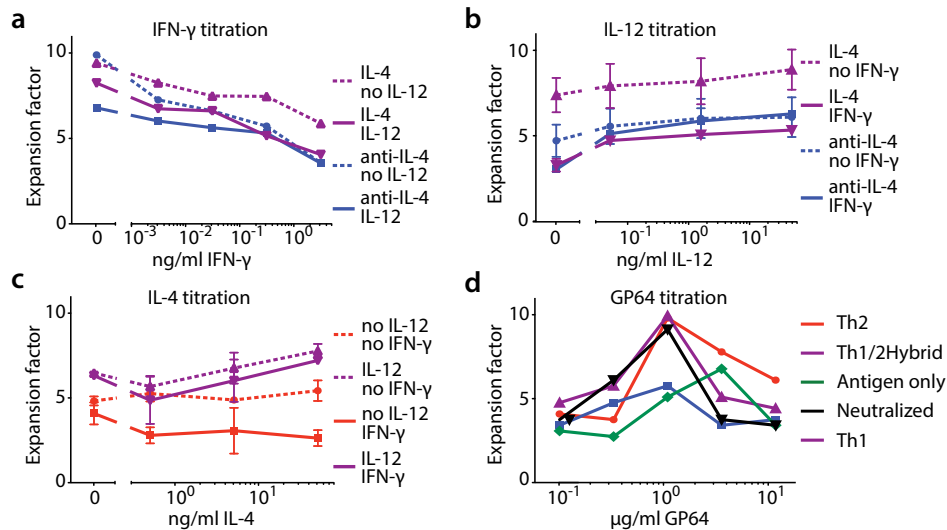
Figure 7.9: IFN-γ reduces cell expansion, whereas IL-12 and IL-4 tend to augment it. **a** Cell expansion in response to IFN-γ with IL-12 (full lines) or without IL-12 (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **b** Cell expansion in response to IL-12 with IFN-γ (full lines) or without IFN-γ (dotted lines), in the presence of IL-4 (purple lines) or anti-IL-4 (blue lines). **c** Cell expansion in response to IL-4 with IFN-γ (full lines) or anti-IFN-γ (dotted lines), in the presence of IL-12 (purple lines) or without IL-12 (red lines). **d** Cell expansion in response to GP64$_{61-80}$ in the different conditions.

7.2 Acknowledgments

7.3   Selbstständigkeitserklärung

Hiermit erkläre ich, Elsa Pellet, geboren am 24.08.1986 in Morges (CH), dass ich die vorliegende Arbeit selbständig verfasst und gemäß §7 Absatz 3 der Promotionsordnung vom 27.06.2012 keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich versichere, dass diese Arbeit in dieser oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegt wurde und ich mich nicht bereits anderwärts um einen Doktorgrad beworben habe bzw. einen entsprechenden Doktorgrad besitze. Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin vom 27.06.2012 ist mir bekannt.

Hannover, den 29.06.2018

7.4   Publication list

1. *In preparation*: **Pellet, E.**, Peine, M., Helmstetter, C., Flossdorf, M., Löhning, M., & Höfer, T. *A densely connected transcription factor network acts as a rheostat that specifies a continuum of T helper cell states.*

7.5   Scientific presentations

1. **Pellet, E.**, Peine, M., Flossdorf, M., Löhning, M., & Höfer, T. *GATA-3 regulation and stability in CD4+ T helper cells.* AAI Annual Meeting, Honolulu, 2013. *Poster presentation*

2. **Pellet, E.**, Peine, M., Helmstetter, C., Flossdorf, M., Löhning, M., & Höfer, T. *GATA-3 regulation and stability in murine T helper cells.* InteGeR, Athens, 2012. *Poster presentation*

3. **Pellet, E.**, Peine, M., Löhning, M., & Höfer, T. *On the role of polarizing cytokines in T helper cell differentiation and modulation of the immune response.* SBHD, Boston, 2011. *Poster presentation*