Universidad Nacional Autónoma de México
Doctorado en Ciencias Biomédicas

Modelación de la diferenciación de células T cooperadoras

Tesis
que para optar por el grado de:
Doctor en Ciencias

Presenta:
Mariana Esther Martínez Sánchez

Tutor principal:
Dra. Elena Alvarez-Buylla
Instituto de Ecología, UNAM

Miembros del comité tutor:
Dra. Marcia Hiriart
Instituto de Fisiología Celular, UNAM
Dr. Luis Mendoza
Instituto de Investigaciones Biomédicas, UNAM

CIUDAD DE MÉXICO
México, D.F. Enero, 2017

Agradecimientos

Gracias, muchas gracias.

Índice general

Índice de figuras

Índice de cuadros

Resumen

Las células T CD4+ orquestan la respuesta inmune adaptativa en vertebrados. Estas células son capaces de diferenciarse en distintos tipos celulares dependiendo de las señales de su entorno, modulando a su vez al resto de la respuesta inmune. Aunque se han llevado a cabo múltiples estudios tanto experimentales como teóricos para entender los mecanismos moleculares implicados, aún no es claro como los componentes moleculares intrínsecos (producidos por la misma célula) se integran de manera dinámica con los componentes extrínsecos (producidos por otras células) para dar origen a los distintos tipos celulares y su plasticidad fenotípica. En este trabajo proponemos una serie de redes Booleanas que integran los datos disponibles de regulación molecular tomando en cuenta su comportamiento dinámico para contribuir al entendimiento integral de la red de regulación que subyace la diferenciación y la plasticidad de las células T CD4+.

En primer lugar, construimos una red de regulación Booleana conformada por los factores transcripcionales maestros implicados en la diferenciación de las células T CD4+. Mostramos que en el modelo estos factores transcripcionales maestros son necesarios, pero no suficientes, para recuperar los fenotipos de las células T CD4+ descritos experimentalmente. A continuación, postulamos una red Booleana regulatoria mínima derivada de una red más amplia basada en datos experimentales. La red mínima integra la regulación transcripcional, las vías de señalización y el microambiente, definido como las citocinas que rodean a la célula. Este modelo reproduce las configuraciones de la mayoría de los tipos celulares caracterizados (Th0, Th1, Th2, Th17, Tfh, Th9, iTreg, Tr1 y Th3). Además, el modelo reproduce los subtipos de células T CD4+ inducidos en diferentes micro-ambientes después de la activación y recupera muchos de los patrones de plasticidad documentados para diferentes tipos de células T CD4+. Proponemos una metodología novedosa para determinar los nodos clave, mostrando que T-bet, TGFB y supresores de las proteínas de señalización de citocinas (SOCS) son nodos claves para reproducir de las respuestas celulares observadas experimentalmente.

También estudiamos cómo el entorno hiperinsulinémico presente en la inflamación crónica asociada a la obesidad afecta a las células T CD4+. Es conocido que la insulina influye en la respuesta inmune a través de su efecto sobre el estado metabólico de las células, pero también regula la expresión de citocinas reguladoras como IL-10. Entender como los cambios metabólicos afectan a los linfocitos T CD4+ puede ayudar a establecer marcos terapéuticos para controlar la inflamación

crónica asociada al síndrome metabólico. En presencia de altos niveles de insulina, poblaciones post-inflamatorias como Th1 y Th17 se vuelven más resistentes a perturbaciones transitorias y su tamaño de cuenca aumenta, las células reguladoras Tr1 se vuelven menos estables o desaparecen, mientras que las células productoras de TGFβno son alteradas. Nuestras simulaciones muestran que en ambientes pro-Th1, pro-Th2 y pro-Th17 pueden coexistir células efectoras y reguladoras, pero que altos niveles de insulina afectan severamente a las células reguladoras, especialmente en un entorno pro-Th17. Por lo tanto, el modelo proporciona una explicación dinámica y sistémica para el papel bivalente de TGFβen la inflamación como en la regulación de la respuesta inmune y en la aparición del fenotipo Treg adiposo.

Las múltiples retroalimentaciones entre los factores transcripcionales, vías de señalización y el medio ambiente tienen un papel importante en la diferenciación y plasticidad de las células T CD4+. Este patrón de vías altamente interconectadas puede observarse en otros modelos biológicos. Para determinar la relación entre la estructura topológica de las interacciones, las funciones que describen su comportamiento dinámico, las funciones y los estados estables o atractores a los cuales converge el sistema, estudiamos sistemáticamente el papel de las combinaciones de circuitos funcionales en pequeñas redes booleanas. Mostramos que las combinaciones de estos circuitos funcionales regulan tanto el número como el tamaño de los atractores. Este análisis permite caracterizar las estructuras reguladoras y su papel en la dinámica del sistema.

Los tipos de células T CD4+ observados y los patrones de transición entre ellos emergen de la retroalimentación entre el núcleo regulador intracelular y el microambiente. La inducción de fenotipos estables y la plasticidad de las células T CD4+ se puede estudiar usando el marco metodológico propuesto aquí. Este enfoque se puede extender para otros sistemas biológicos plásticos. Las herramientas desarrolladas aquí están disponibles para el público. Este trabajo proporciona un marco formal, dinámico y sistémico para integrar datos experimentales en el estudio de enfermedades inflamatorias complejas.

Abstract

CD4+ T cells orchestrate the adaptive immune response in vertebrates. While both experimental and modeling work has been conducted to understand the molecular genetic mechanisms involved in CD4+ T cell responses and fate attainment, the dynamic role of intrinsic (produced by CD4+ T lymphocytes) versus extrinsic (produced by other cells) components remains unclear. Furthermore, the mechanistic and dynamic understanding of the plastic responses of these cells remains incomplete.

We first show that the regulatory core composed of master transcription factors is not sufficient to recover common CD4+ T phenotypes. We thus postulate a minimal Boolean regulatory network model derived from a larger and more comprehensive network that is based on experimental data. The minimal network integrates transcriptional regulation, signaling pathways and the microenvironment. This network model recovers reported configurations of most of the characterized cell types (Th0, Th1, Th2, Th17, Tfh, Th9, iTreg,Tr1, and Th3). Additionally, this model recovers many of the plasticity patterns documented for different T CD4+ cell types, as summarized in a cell-fate map.

We tested the effects of various micro-environments and transient perturbations on such transitions among CD4+ T cell types. We propose a novel methodology to determine key nodes, showing that the nodes T-bet, TGF$\beta$and suppressors of cytokine signaling proteins (SOCS) are key to recovering observed CD4+ T cell plastic responses.

We also study how the hyperinsulinemic environment present in obesity associated chronic inflamation affects CD4+ T cell attainment. In the presence of high levels of insulin Th1 and Th17 become more stable to transient perturbations and their basin sizes are augmented, Tr1 cells become less stable or disappear, while TGFβproducing cells remain unaltered. Our simulations show that in pro-Th1, pro-Th2 and pro-Th17 environments effector and regulatory cells can coexist, but that high levels of insulin severely affect regulatory cells, specially in a pro-Th17 environment. Hence, the model provides a dynamic system-level explanation for the bivalent role of TGFβin both inflammation and regulation of immune responses and the emergence of the adipose Treg phenotype.

The multiple feedback loops between transcription factors, signaling pathways and the environment has an important role in CD4+ T cell attainment. This pattern of highly interconected

pathways can be observed in other biological models. To determine the relationship between topology, functions and attractors, we systematically study the role of the combinations of functionalites in small Boolean networks. We show that the combinations of functionalities regulate both the number and size of the attractors. This analysis lets us characterize the regulatory structures and their role in the dynamics of the system.

The observed CD4+ T cell-types and transition patterns emerge from the feedback between the intrinsic or intracellular regulatory core and the micro-environment. CD4+ T cell stability and plasticity can be studied using the methodological framework proposed here. This approach can be used for other plastic systems, an the tools developed here are available to the public. This work provides a system-level formal and dynamic framework to integrate further experimental data in the study of complex inflammatory diseases.

Introducción

Los organismos viven en un entorno cambiante. En algunos casos, los organismos deben ignorar estos cambios y mantener un fenotipo estable, mientras que en otros deben reaccionar cambiando su fenotipo. Distinguir entre estas dos situaciones no es trivial, ya que los seres vivos requieren considerar su estado interno y las señales ambientales que los rodean. Esto significa que para sobrevivir los organismos requieren fenotipos que sean a la vez estables y plásticos. Cómo los mecanismos de regulación molecular les permiten a los seres vivos tener fenotipos robustos es una pregunta abierta.

El sistema inmune ejemplifica cómo los organismos requieren al mismo tiempo estabilidad y plasticidad. El sistema inmunitario defiende al organismo contra una amplia gama de patógenos y desafíos inmunes. Para controlar completamente un patógeno, el sistema inmunológico debe mantener una respuesta hasta que el patógeno ha sido eliminado, si esto falla se pueden desarrollar infecciones crónicas. Sin embargo, conforme la infección y la respuesta inmune progresan las circunstancias cambian, de tal forma que el sistema inmune debe adecuarse a ellas. Una vez superado el desafío inmune, el sistema inmune se autoregula para evitar enfermedades autoinmunes. De esta manera, para mantener la homeostasis es necesaria una respuesta estable que elimine a los patógenos y suficiente plasticidad para adaptarse a los cambiantes desafíos inmunológicos [1].

Las células T CD4+ son parte de la respuesta inmune de adaptación y ayudan a coordinar las diferentes partes de la respuesta inmune. Cada tipo de célula activa o inhibe diferentes ramas de la respuesta inmune [2]. Las células T CD4+ se diferencian en respuesta a las citocinas en su microambiente, las cuales pueden ser producidas por la misma célula (intrínseca) o por otras células del organismo (extrínsecas). Las citocinas se unen a receptores de membrana y activan cascadas de señalización que terminan con la translocación de factores de transcripción al núcleo [3]. Hay regulación entre las vías a través de proteínas SOCS, que compiten con factores de transcripción como STAT para el sitio de fosforilación en el receptor [4, 5, 6, 7]. Una vez que la señal ha llegado al núcleo, los factores de transcripción pueden activar o inhibir otros factores de transcripción y citocinas, configurando la diferenciación en los diferentes subconjuntos [8, 9, 10]. Este control genético también está influenciado por otros factores como las marcas epigenéticas y el metabolismo [8, 11]. Las citocinas producidas por la célula son secretadas en el entorno, donde se unen a las citocinas

producidas por otras células del sistema inmune y del organismo. Las señales en el microambiente son fundamentales para la determinación y mantenimiento del destino celular [12, 13, 14, 15].

Las células T CD4+ tienen un perfil transcripcional heterogéneo y pueden trans-diferenciarse en respuesta a los cambios en el microambiente [16, 17, 18, 19]. También hay una considerable superposición entre los perfiles de expresión de diferentes células T CD4+. Existen informes de células híbridas Treg/Th17, Treg/Th1 e incluso Th1/Th2 [20, 21, 22]. La citocina reguladora IL-10 puede ser secretada por células Th1, Th2, Th17, iTreg y una variedad de otras células inmunitarias [23, 24].

Una vez diferenciadas, las células T CD4+ pueden cambiar dinámicamente su patrón de expresión conforme los retos inmunológicos y las señales del entorno cambian. Estas transiciones plásticas entre tipos celulares se han asociado con el mantenimiento de la homeostasis y con algunas enfermedades. Por ejemplo, la transición de Treg a Th17 se ha asociado con respuesta antitumoral, pero también con esclerosis múltiple y psoriasis [25]. Al mismo tiempo, existen restricciones a esta plasticidad, algunas transiciones son más comunes como la transición Treg/Th17, mientras que otras parecen ser poco comunes como la transición Th1/Th2 [25, 18, 19].

Las células T CD4+ tienen patrones de expresión altamente heterogéneos además de plasticidad fenotípica [16, 17, 18, 19]. Es por esto que una pregunta importante es si los tipos celulares observados experimentalmente -y sus transiciones asociadas- son linajes o subtipos [19]. El término linaje implica compromiso con un patrón de expresión y estabilidad fenotípica ante alteraciones ambientales. Por otro lado, el término subconjunto implica que, aunque la célula tiene un patrón de expresión especificado, depende de señales extrínsecas para mantenerlo [19]. Determinar si los tipos de células CD4+ son subtipos, linajes o algo intermedio requiere un enfoque sistémico que estudie el comportamiento dinámico de cada tipo celular en relación con otros tipos celulares y el microambiente.

Las células T CD4+ también interactuan con el resto del organismo, en particular, el metabolismo regula fuertemente a las células T CD4+ [11]. La inflamación crónica asociada a la obesidad (ICAO) se caracteriza por la retroalimentación entre la respuesta inflamatoria del sistema inmune y el metabolismo que se ve alterado en la obesidad. En la ICAO hay un aumento en las células Th1 y Th17 inflamatorias, y una disminución en la producción de Tregs e IL10 [26, 27, 28]. La hiperinsulinemia, que está asociada con la obesidad y el síndrome metabólico, inhibe la IL-10 y disminuye el número y la estabilidad de las células Treg[29]. Comprender cómo la insulina afecta la regulación de las células T CD4+ nos ayuda a entender la relación entre la respuesta inmune y el metabolismo.

Definir el fenotipo de las células T CD4+ no es trivial, ya que hay que tener en cuenta los perfiles transcripcionales heterogéneos, la respuesta dinámica al medio ambiente, las transiciones plásticas entre los tipos celulares y su relación con el resto del organismo. Este comportamiento celular dinámico de las células T CD4+ es el resultado de una compleja red reguladora de factores de transcripción, vías de señalización, citocinas extracelulares. Entender cómo esta red reguladora subyace en el logro del destino de las células y permite que las células T CD4+ mantengan su

función frente a un entorno cambiante puede ayudarnos a entender no sólo la respuesta inmune, sino cómo los seres vivos logran robustez.

Entender el comportamiento de las células CD4+ requiere una estrategia de modelado que integre la información de la regulación del sistema para dar una explicación mecánica de los comportamientos celulares observados. Para ello, es necesario un modelo que sea capaz de incluir múltiples tipos de moléculas y captar las interacciones clave, tomando en cuenta que la respuesta inmune es dinámica y fuertemente influenciada por el entorno. El modelo resultante debe ser capaz de recuperar los patrones de diferenciación observados, la plasticidad y la robustez del sistema, ser comprensible y hacer predicciones que pueden ser validadas experimentalmente.

La herramienta de modelación más sencilla que satisface estos requisitos son las redes booleanas [30]. Las redes booleanas integran la información disponible de la regulación molecular para predecir fenómenos de nivel celular utilizando un formalismo matemático de funciones booleanas. Estas redes consisten en nodos -que representan genes, proteínas y otros procesos biológicos- e interacciones -que representan las interacciones reguladoras entre los nodos-. Como las redes booleanas son dinámicas, es posible construir funciones que describan el estado de los nodos dependiendo del estado de sus reguladores a través del tiempo. El valor del nodo representa si el gen o proteína esta activo o inactivo funcionalmente en el sistema biológico. El efecto del entorno se puede incluir en estos modelos como nodos de entrada. Las funciones boolenas se evalúan para obtener los atractores de la red, que representan tipos celulares o procesos biológicos como el ciclo celular [31, 32, 33]. Además, las redes booleanas nos permiten simular múltiples tipos de perturbaciones, lo que las hace ideales para estudiar el destino celular [34, 35]. Las redes booleanas se han utilizado ampliamente para estudiar cómo los fenotipos celulares emergen a partir de la regulación molecular[30].

La complejidad del sistema y el número de moléculas implicadas en la respuesta inmune es suficientemente grande para que el análisis de todas las moléculas implicadas no sea trivial. Una pregunta que surge de este problema es determinar cuáles de estos niveles regulatorios y moléculas son necesarios y suficientes para modelar el fenotipo. Un nodo es necesario si es imposible explicar el fenotipo sin él. Al mismo tiempo, que una molécula sea necesaria no significa que sea suficiente, ya que puede requerir la colaboración de otras moléculas [31, 32]. Determinar el conjunto de moléculas que son necesarias y suficientes para recuperar los fenotipos esperados nos ayuda a determinar si nuestro conocimiento es suficiente para entender el sistema y además de que nos permite obtener un modelo capaz de hacer predicciones globales del comportamiento del sistema.

La determinación del destino celular de los linfocitos T CD4+ requiere múltiples niveles de regulación y las interacciones entre ellos. Las asas de retroalimentación y los circuitos funcionales son fundamentales en la conformación del comportamiento dinámico del sistema [36, 37]. Un asa de retroalimentación es un conjunto de interacciones dirigidas que forman una trayectoria cerrada, y pueden ser positivas o negativas, dependiendo de los signos de sus interacciones [38, 39]. Estas asas de retroalimentación se encuentran en la auto-inducción de los factores de transcripción, la inhibición mutua entre los factores de transcripción, los circuitos de retroalimentación positiva entre los factores de transcripción y citocinas o en la inhibición entre las vías de señalización [36, 37, 30].

Comprender la estructura de estas estructuras y su función en la dinámica del sistema es importante para entender cómo las redes reguladoras biológicas mantienen la estabilidad -y la plasticidad- de las células T CD4+.

Para entender la robustez del sistema inmunológico es necesario estudiar tanto la estabilidad como la plasticidad de las células T CD4+ en un contexto global. La robustez es la capacidad de un organismo de mantener su función biológica en respuesta a las perturbaciones. Un sistema es estable si regresa a un estado dado después de una perturbación, y plástico si transita a uno nuevo. Tanto la estabilidad como la plasticidad son necesarias para mantener la homeostasis. Sin embargo, para estudiar la robustez es necesario determinar qué función del sistema es robusta a qué tipo de perturbaciones [40]. Además, es importante desarrollar métodos para cuantificar esta robustez y determinar los componentes clave del sistema.

Las células T CD4+ están sujetas a un entorno cambiante, y su regulación interna puede verse afectada por el ruido durante el desarrollo, las mutaciones y otras perturbaciones que alteran la regulación molecular [41]. Los modelos booleanos nos permiten estudiar la robustez, estudiar el efecto de pérdida y ganancia de función, los cambios en el microambiente y verificar nuestro modelo usando los diversos tipos de información biológica disponible. Estos modelos también pueden usarse para estudiar el efecto de las perturbaciones transitorias en los componentes intrínsecos de la red y las entradas del sistema. Los modelos booleanos nos permiten comprobar si hay sobreajuste, el posible efecto de errores en la construcción de las funciones, y predecir las interacciones faltantes [42].

Hipótesis y objetivos

Hipótesis

La diferenciación y la plasticidad de las células T CD4+ cooperadoras emerge de una red de regulación molecular que incluye factores transcripcionales, vías de señalización y citocinas.

Objetivo general

Generar un modelo de redes Booleanas que sea capaz de recuperar tanto los tipos celulares como el comportamiento dinámico de las células T CD4+ cooperadoras.

Objetivos particulares

Las células T CD4 + tienen un papel crítico en el organismo, integrando las señales del entorno para coordinar la respuesta inmune. Estas células han sido ampliamente estudiadas por los inmunólogos, tanto a nivel molecular como celular, sin embargo, aún no entendemos como los comportamientos a nivel célular emergen de la regulación molecular. Hoy en día es posible utilizar las herramientas de modelado desarrolladas por matemáticos e informáticos para integrar la información disponible. Utilizando un enfoque sistémico dinámico podemos comenzar a resolver algunas de las preguntas abiertas que rodean el logro de destino de células T CD4 +.

Primero, el artículo .ª Minimal Regulatory Network of Extrinsic and Intrinsic Factors Recovers Observed Patterns of CD4+ T Cell Differentiation and Plasticityütilizamos un enfoque interdisciplinario para estudiar la diferenciación y la plasticidad de las células T CD4 +. En primer ligar determinamos los elementos que debe contener la red de reglación mínima. Utilizando esta red determinamos que tipos célulares se pueden recuperar y su robustez ante cambios en el entorno y perturbaciones transitorias. En este primer artículo abordamos las siguientes preguntas:

- ¿Cómo surge la plasticidad y la estabilidad a partir de la red de regulación?
- ¿Qué niveles reguladores -factores transcripcionales, vías de señalización, citocinas- son necesarios y suficientes para recuperar el comportamiento de las células T CD4 +?

- ¿Cuál es el papel del ambiente en el comportamiento de las células T CD4 +?
- ¿Los tipos de células T CD4 + reportados son subtipos o linajes?

Segundo, el artículo "The CD4+ T cell regulatory network mediates inflammatory responses during acute hyperinsulinemia: a simulation study.[es]tudiamos la relación entre las células T CD4 + y el metabolismo, centrándonos en el papel de la hiperinsulinemia y sus implicaciones para la ICAO. Este artículo presenta una primer intento de una aproximación para integrar diferentes sistemas biológicos para estudiar las enfermedades complejas. En este segundo artículo abordamos las siguientes preguntas:

- ¿Cómo podemos modelar la relación de las células T CD4 + con el resto del metabolismo?
- ¿Cómo altera la hiperinsulinemia la red reguladora de células T CD4 + para generar los tipos celulares observados en ICAO?

Los enfoques interdisciplinarios no sólo enriquecen la biología, sino que también mejoran las herramientass de modelación y nos dan una idea de las propiedades de los sistemas dinámicos. Comprender las propiedades de las herramientas de modelación nos ayuda a entender las ventajas y limitaciones de los modelos, motivando el desarrollo de nuevas herramientas e ideas tanto biológicas como matemáticas.

Tercero, el artículo "The combination of the functionalities of feedback circuits is determinant for the number and size of attractors of molecular networks.[es]tudia la relación entre la topología y las funciones de las redes booleanas. Este artículo utiliza la comprobación de modelos para determinar cuáles son los factores que determinan la dinámica de una red. En este tercer artículo abordamos la pregunta:

- ¿Cuál es la relación entre la topología, las funciones y los atractores de una red Booleana?
- ¿Cuál es el papel de las asas de retroalimentación y los circuitos funcionales en el sistema?

Cuarto, el tutorial para el paquete R "BoolNetPerturb: Robustness and Plasticity in Regulatory Networks". BoolNetPerturb extiende el paquete de R BoolNet para permitir la simulación sistemática de las perturbaciones en las redes booleanas. Esta herramienta está disponible públicamente en github bajo una licencia Creative Commons. En esta cuarta sección abordamos las siguientes preguntas:

- ¿Qué perturbaciones pueden ser modelar utilizando redes booleanas?
- ¿A que procesos biológicos corresponden estas perturbaciones?
- ¿Cómo medir la robustez y determinar los nodos clave de un sistema?

Esperamos que un enfoque a nivel de sistema que integre los diferentes niveles de regulación y tenga en cuenta la heterogeneidad de los tipos celulares y su comportamiento dinámico puede ayudar a entender el rico comportamiento de las células T CD4 + y la robustez de la respuesta inmune.

Capítulo 1

Marco teórico

1.1. El sistema inmune

El sistema inmune es el encargado de proteger al organismo de infecciones y mantener la homeostasis del cuerpo. Existe una gran variedad de patógenos que pueden afectarnos: virus, bacterias, hongos, helmintos; además existen retos generados por el mismo organismo, como las células cancerosas o las enfermedades autoinmunes causadas por la desregulación del mismo sistema inmune [43].

En primer lugar, el sistema inmune reconoce al patógeno y es necesario detectar con absoluta especificidad a los patógenos y distinguirlos de las células normales del organismo [43]. Una vez que el patógeno ha sido reconocido, el sistema inmune monta una respuesta que permita eliminarlo o neutralizarlo, si el sistema inmune fracasa en montar la respuesta necesaria por el tiempo adecuado es incapaz de controlar al patógeno, llevando a infecciones crónicas [43]. Una vez que el sistema inmune ha controlado al patógeno la respuesta inmune debe de ser controlada para evitar que dañe al mismo organismo, por lo que existen mecanismos de regulación de la respuesta inmune que ayudan a recuperar la homeostasis del organismo [43].

A lo largo del tiempo han evolucionado múltiples mecanismos celulares y moleculares para llevar a cabo estas funciones. Estos mecanismos se dividen en dos tipos principales: innatos y adaptativos [Fig: 1.1], cuya diferencia principal reside en las estructuras de reconocimiento de los patógenos y su tiempo de acción [43]. Además, en años recientes se ha encontrado una gran riqueza de células reguladoras que controlan a la misma respuesta inmune [43, 44, 45].

1.1.1. Inmunidad innata

El sistema inmune innato o no específico se refiere a los mecanismos básicos de resistencia de un organismo. En lugar de reconocer específicamente al patógeno, la respuesta inmune innata reconoce patrones moleculares altamente conservados comunes a un grupo o familia entera de patógenos.

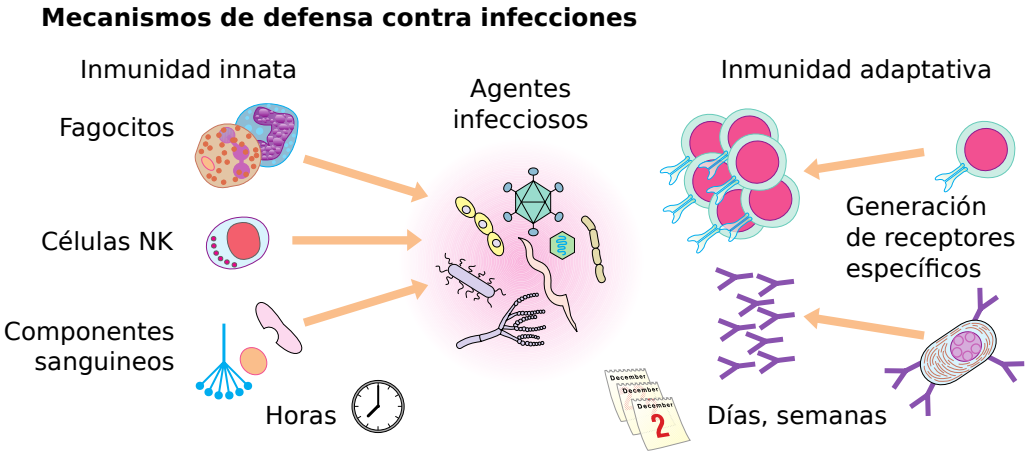


Figura 1.1: El sistema inmune consta de la inmunidad innata y la adaptativa. La inmunidad innata reconoce patrones conservados y actúa rápidamente. Mientras tanto, la inmunidad adaptativa es específica y puede tardar días en montar una respuesta. (tomada de [46])

y

Estos mecanismos conforman la respuesta inmediata a los patógenos y se mantienen a lo largo de toda la reacción inmunitaria [43].

Existen cuatro tipos de barreras: anatómicas, fisiológicas, endocíticas y fagocíticas e inflamatorias [43].

- Las barreras anatómicas impiden físicamente la entrada de patógenos al organismo. Éstas incluyen la piel y las membranas mucosas.
- Las barreras fisiológicas incluyen temperatura, pH, oxígeno y otros factores solubles. Éstas actúan generando ambientes poco favorables para el patógeno.
- Las barreras endocíticas y fagocíticas internalizan y degradan macromoléculas extracelulares dentro de compartimientos específicos de la célula. La mayoría de las células son capaces de endocitosis; los monocitos, neutrófilos y macrófagos se especializan en la fagocitosis.
- Las respuestas inflamatorias incrementan el flujo sanguíneo, la permeabilidad capilar y la cantidad de células fagocíticas y proteínas de fase aguda.

1.1.2. Inmunidad adaptativa

La inmunidad adaptativa o específica se caracteriza por tener especificidad, diversidad, memoria y reconocimiento de lo propio y ajeno. La especificidad puede reconocer y responder a una amplia gama de moléculas blanco de manera individual. Su diversidad le permite reaccionar a distintos antígenos. La memoria puede alterar las respuestas a partir de experiencias previas, montando una

respuesta más rápidamente. Finalmente el reconocimiento de lo propio y ajeno le permite diferenciar lo propio de lo ajeno[43].

La respuesta inmune adaptativa no es inmediata, ya que requiere tiempo para reconocer los retos inmunológicos y montar una respuesta. La respuesta innata desencadena las primeras señales que activan a la inmunidad adaptativa, la cual regula y dirige factores específicos de la inmunidad innata aumentado su eficiencia. La activación de la respuesta inmune adaptativa depende de la interacción entre las células presentadoras de antígeno (APC) y los linfocitos[43].

1.1.3. Hematopoyesis

La hematopoyesis es el proceso de producción de células como eritrocitos, neutrófilos, monocitos, linfocitos, entre otras, las cuales se encuentran presentes en la sangre y otros tejidos del organismo. La diferenciación de las células hematopoyéticas empieza por la célula madre hematopoyética multipotente, la cual puede dar origen a dos linajes, el mieloide y el linfoide. El linaje mieloide incluye a los megacariocitos, eritrocitos neutrófilos, monocitos, macrófagos, células dendríticas entre otras. El linaje linfoide incluye a células NK, B y T entre otras[47, 48, 49].

Las primeras células linfoides de un embrión humano aparecen en el tejido hematopoyético, aproximadamente a los dos meses de vida intrauterina. Las células B y T se generan a partir de un subgrupo de células totipotenciales de la médula ósea que expresan Notch. Notch-1 induce al progenitor tímico temprano (ETP), a partir del cual derivarán los linfocitos T. En ausencia de Notch1 se induce al progenitor linfoide temprano (ELP), derivándose los linfocitos B [50].

Una vez en el timo, ETP inicia el rearreglo y la expresión de los genes β, γ y δ del receptor de células T (TCR). En este momento los linfocitos inmaduros no expresan a CD4 ni a CD8, por lo que se les llama linfocitos pro-T o T CD4-CD8-. Una minoría de estas células expresa los receptores $\gamma\delta$ y se diferencia en linfocitos T $\gamma\delta$. El resto de los linfocitos expresan las cadenas $\alpha\beta$ del TCR y se diferencian en linfocitos pre-T o en células doblemente positivas CD4+CD8+[43].

A continuación los linfocitos CD4+CD8+ pasan por la selección positiva, que consiste en unirse con baja afinidad a los péptidos presentados por el MHC. Los linfocitos con afinidad al MHC I se diferenciaran en células T citotóxicas o CD4-CD8+. Los linfocitos con afinidad por el MHC II se diferenciaran en células T cooperadoras o CD4+CD8-. Posteriormente los linfocitos pasan por la selección negativa, donde las células que reconocen con alta avidez o afinidad los péptidos propios son eliminadas, asegurándose así la destrucción de células potencialmente reactivas[43]. Algunas de las células que reconocen antígenos propios se diferencian en nTreg. Las células nTreg expresan tanto CD4 como Foxp3, son células reguladoras que inhiben la respuesta inmune favoreciendo la tolerancia a antígenos propio[51, 52].

Las células T$\alpha\beta$ con un solo marcador CD4+ o CD8+ salen a los tejidos linfoides periféricos donde podrán ser activadas, proliferar y diferenciarse[43] [Fig: 1.2].

Una vez que las células T CD4+ han madurado, migran al sistema linfático y circulatorio como células vírgenes Th0, donde son expuestas a diversos antígenos[Fig: 1.3]. La mayoría de los antígenos son transportados al sistema linfático donde son procesados por las APCs, que expresan

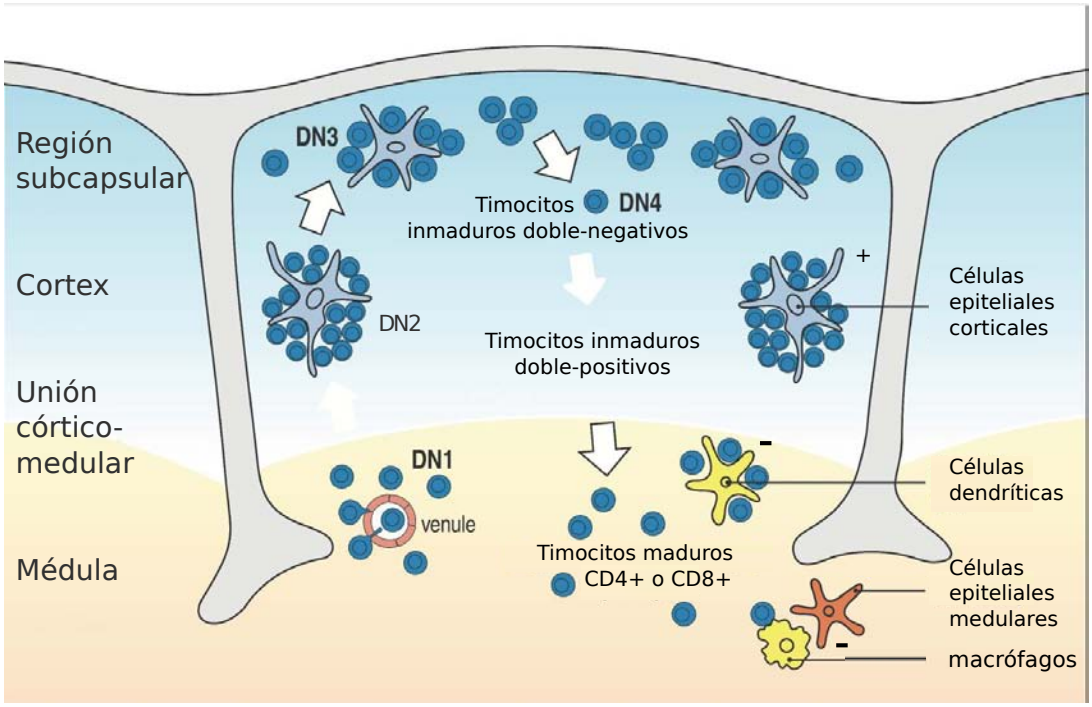


Figura 1.2: Maduración y selección de los linfocitos T. Los linfocitos T maduran en el timo, donde pasan por la selección negativa y positiva. (tomada de [53])

los antígenos junto con el MHC II en membrana. La alta variabilidad sus receptores hace que pocos sean específicos para cada patógeno. Una vez activados, los linfocitos proliferan para incrementar su población. Este periodo de proliferación requiere al menos una semana para desarrollarse, durante la cual el control de la infección por los mecanismos innatos es esencial para la supervivencia del individuo. El reconocimiento del MHC por el TCR activa la expresión de factores transcripcionales como NFAT y NF-κB, que son vitales en la activación y proliferación de las células T CD4+[43]. Una vez activados los linfocitos se multiplican clonalmente, diferenciándose en distintos tipos celulares dependiendo de la señalización del TCR y las citocinas presentes en el ambiente determinan la diferenciación de la célula T CD4+. Las células efectoras llevan a cabo la respuesta inmune contra el patógeno y regulan a otras células del sistema inmune. Estas células tienen una vida corta, ya que una respuesta inmune excesiva puede dañar al organismo. Las células de memoria se replican menos, viven largo tiempo y sirven para incrementar la velocidad e intensidad de la respuesta inmune si se vuelve a encontrar al mismo patógeno[43].

1.2. Células T CD4+

Las células T CD4+ tienen un papel fundamental en el sistema inmune. Estas células censan la información en su micro-entorno, se diferencian en distintos tipos celulares y regulan a las distintas

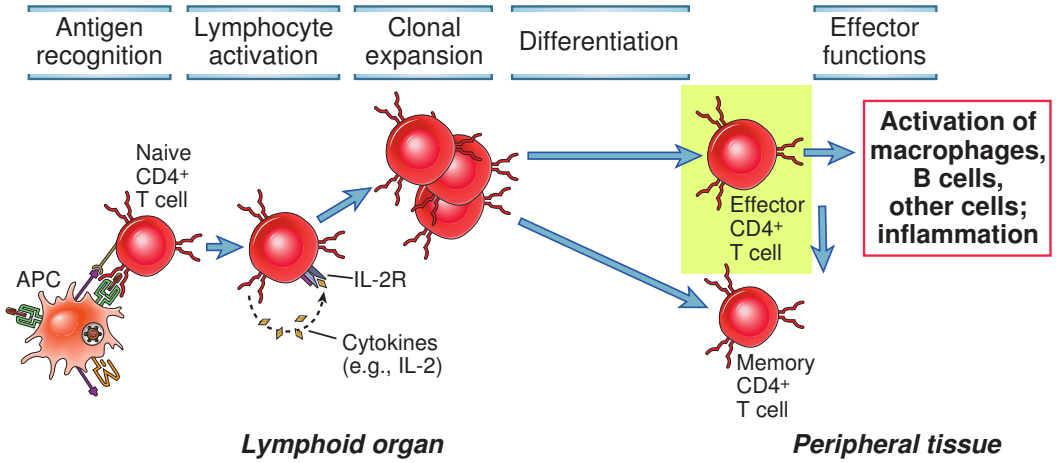


Figura 1.3: Activación de los linfocitos T. Los linfocitos T CD4+ maduros son activados al reconocer el antígeno presentado por CPA. Una vez activados se expanden clonalmente y diferencian en distintos tipos celulares, cada uno asociado con diferentes funciones. (tomada de [54])

ramas del sistema inmune. Las células T CD4+ que salen del timo al resto del organismo se conocen como células vírgenes o Th0, ya que aún no han conocido antígeno. Una vez activadas, empieza una cadena de señales moleculares y cambios en la expresión de factores de transcripción que lleva a la diferenciación en distintos tipos celulares[43] [Fig: 1.4].

Originalmente, estas células se clasificaron en dos grandes grupos a partir de su patrón particular de producción de citocinas y de funciones: los linfocitos Th1 encargados de la respuesta celular y los linfocitos Th2, encargados de la respuesta humoral[55]. En 2006 se definieron los linfocitos Th17, asociadas con respuestas inflamatorias y anti-tumoral[56]. Pronto, se empezaron a describir mas y mas tipos celulares como Th9 asociadas con respuesta a gusanos parasíticos[57, 58, 59, 60], los linfocitos Th22 asociadas con la respuesta inmune en la piel[61, 62, 63], y los linfocitos Tfh asociadas con la formación de centros germinales y maduración de linfocitos B[64, 65, 66, 67, 68].

Las células T CD4+ no solo están implicadas en la defensa contra patógenos, también se han asociado con la regulación de la respuesta inmune, siendo fundamentales para mantener la tolerancia y la homeostasis del organismo. Originalmente, células con esta función se reportaron en los 70's, pero dificultades experimentales hicieron que se creyera que eran un error[45, 69]. Sin embargo, en 1995 se definieron a las células reguladoras Treg como encargadas de controlar las respuestas autoinmunes[70]. Actualmente se han reportado células Tr1 Foxp3-IL10+ asociadas con y células Th3 Fox3-TGFβ+ asociadas con a la regulación de la inflamación y la tolerancia oral[71, 72, 22, 73].

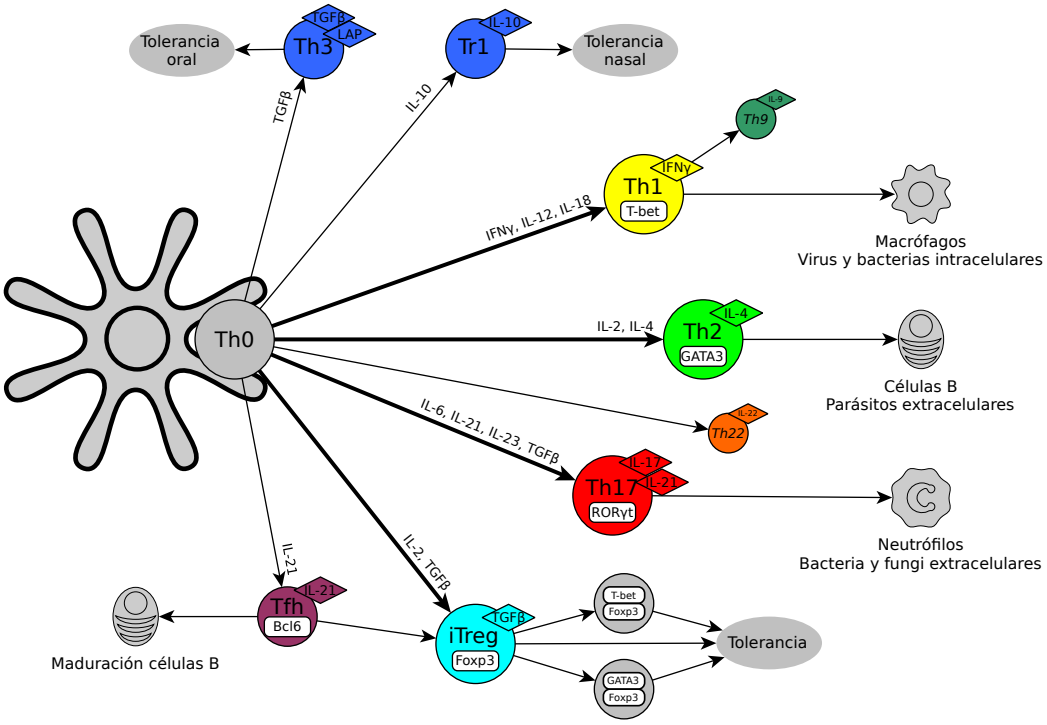


Figura 1.4: Las células Th0 pueden diferenciarse en distintos tipos celulares como: Th1, Th2, Th17, Th9, Th22, Tfh, Treg, Th3, Tr1. Cada linaje es inducido por citocinas específicas en el entorno y expresa un patrón de factores transcripcionales y citocinas particular.

1.2.1. Tipos de células

Th0

Las células Th0 o vírgenes son células Th CD4+ a las que aún no se les ha presentado un péptido y por lo tanto no han sido activadas ni se han diferenciado. La cantidad y calidad de células Th0 se ven alteradas en síndromes como el VIH y CVID (Common Variable Immunodeficiency) [74].

Las células Th0 expresan una amplia gama de citocinas. Para la mayoría de los ensayos *in vitro* se supone que las células Th0 no producen citocinas características como IL-4, IFNγ, IL-17, IL-10 y TGFβ. Sin embargo, se han reportado células Th0 que expresan combinaciones de IFNγ, IL-4, IL-5, IL-6, IL-10, IL-17 y GM-CSF [75] las cuales se relacionan generalmente con células Th1, Th2, Th17 y Treg.

Este patrón de expresión de citocinas podría deberse a la regulación de las proteínas SOCS. En linfocitos Th0 la expresión de SOCS3 disminuye, mientras que la expresión de SOCS1 y SOCS2 aumenta. Esto sugiere que la regulación de las proteínas SOCS es diferente entre los linfocitos Th0 y los linfocitos activos, llevando a diferentes patrones de represión de citocinas [76].

Las células Th0 expresan una serie de moléculas de membrana como CD45RA y CD62L. También carecen de marcadores de activación como CD25, CD44, CD62L y CD69. IL-7 y su transductor STAT5 es necesario para la supervivencia de las células Th0, IL-2 favorece la diferenciación hacia células efectoras e IL-15 hacia células de memoria [77].

Th1

Las células Th1 median las respuestas contra patógenos intracelulares como *Mycobacteria*, favorecen la proliferación de células Tc, el reclutamiento de macrófagos y el incremento en su actividad microbicida. Además, está asociado con una serie de enfermedades autoinmunes como la hipersensibilidad tipo 4, esclerosis múltiple y artritis reumatoide.

Los principales productos de las células Th1 son IFNγ, linfotoxina α (LTα) e IL-2. IFNγ fomenta la activación de macrófagos. LTα está relacionada con respuestas inflamatorias, antivirales y autoinmunes como la progresión de la esclerosis múltiple. IL-2 es importante en la maduración de células de memoria CD4 y CD8, además de la diferenciación de las células Th2.

T-bet es el regulador maestro de las células Th1, fomentando la expresión de IFNγ y LTα. T-bet puede ser activado por NFAT y NF-κB junto con STAT1, transductor de IFNγ o por si mismo [78], formando un ciclo de autoinducción. IFNγ es fomentado por T-bet y en menor medida por Eomes. STAT4, el transductor de IL-12 sinergiza con la señal de IL-18 para producir IFNγ. T-bet es un remodelador de la cromatina de IFNγ y se necesita de la señalización del TCR para que IFNγ sea expresado [78]. Las células Th1 sobreregulan IL-12Rβ2 en respuesta a la activación del TCR y de IFNγ. (La cadena IL-12Rβ1 es expresada constitutivamente por las células Th). IL-18Rα es también sobreexpresada en la diferenciación de células Th1. IL-18 e IL-12 sinergizan para expresar IFNγ, lo cual podría explicar la sobreregulación de sus receptores.

Th2

Las células Th2 median las respuestas contra patógenos extracelulares como helmintos. Favorecen la proliferación de células B y el cambio de isotipo a IgE. El tipo está asociado con una serie de enfermedades autoinmunes como la hipersensibilidad tipo 1 o alergia mediada por anticuerpos y el asma.

El principal producto de las células Th2 es IL-4, aunque también producen IL-5, IL-9, IL-13, IL-25 y anfiregulina. IL-4 favorece el cambio de isotipo a IgE y la diferenciación en Th2. IgE se une a basófilos y mastocitos a través de FcϵRI, provocando que secreten histaminas, serotonina, IL-4, IL-12 y TNF-α. IL-5 recluta eosinófilos. IL-9 induce la producción de mucinas durante las reacciones alérgicas. IL-13 fomenta la expulsión de helmintos y la hipersensibilidad de vías respiratorias. IL-25 pertenece a la familia de IL-17 y participa en la amplificación de la respuesta Th2.

GATA-3 es el regulador maestro de las células Th2 y es inducido por STAT6, el transductor de IL-4. También IL-4 incrementa la expresión de GATA-3. A su vez, GATA-3 es capaz de fomentar su propia expresión, además de estar asociado con la elección de linaje a nivel del timo, que está

especialmente expresado en la población transitiva CD4+CD8*bajo*. La sobreexpresión de esta proteína en el timo fetal favorece la polarización hacia CD4, mientras que su eliminación favorece la diferenciación a CD8. IL-4 puede activarse a si mismo a través de su transductor STAT6. GATA-3 es necesario pero no suficiente para fomentar la inducción de IL-4, ya que sólo modifica la cromatina del locus de IL-4 [78]. Es necesaria la ayuda de NFAT, NF-κB, c-Maf o el transductor de IL-2, STAT5[79, 80] para lograr la expresión de IL-4. Por otro lado GATA-3 es necesario y suficiente para lograr la expresión de IL-5 e IL-13. IL-4 induce a Gfi-1. IL-4Rα es sobreregulado por IL-4. CD25 o IL-2Rα es también sobreregulado en las células Th2, posiblemente por la acción de c-Maf. Esto puede incrementar la sensibilidad de las células Th2 a IL-2. IL-33Rα es el principal marcador de superficie de las células Th, su función podría ser análoga a la de IL-18Rα en las células Th1.

Th17

Las células Th17 median las respuestas contra bacterias extracelulares y hongos. Favorecen la respuesta inflamatoria, el reclutamiento de neutrófilos y la respuesta antitumoral. Están asociadas con una serie de enfermedades autoinmunes como la encefalomielitis, psoriasis, artritis reumatoide e inflamaciones gástricas[81].

Los principales productos de las células Th17 son IL-17a, IL-17f, IL-6, IL-21 e IL-22. IL-17a y IL-17f se encuentran en el mismo locus y generalmente son coexpresados y se unen al mismo receptor IL-17RA, aunque IL-17a tiene una mayor afinidad, en general se refiere a ambos como IL-17. Ambos inducen diversas citocinas inflamatorias como IL-6 además de reclutar y activar neutrófilos. IL-21 activa células Tfh, Tc, B, NK, dendríticas y fomenta la diferenciación de Th17. IL-22 fomenta la inflamación dérmica, y la defensa contra bacterias como *K. pneumoinae*, aunque esta ultima podría ser inducida por IL-23.

RORγt es el regulador maestro de las células Th17 e induce IL-17 y a IL-21. RORγt es inducido por STAT3 y por el transductor de IL-1. Sin embargo, existen reportes que indican que RORγtno es necesario para la diferenciación de Th17[82]. También es inducido por bajas concentraciones de TGFβ, pero concentraciones altas de este lo inhiben, aunque no es claro si es directamente o indirectamente a través de Foxp3. RORα también es capaz de inducir IL-17 aunque en menor medida que RORγt. IL-6, IL-21, e IL-23 son todas citocinas pro-inflamatorias que señalizan por medio de STAT3. Además, STAT3 es indispensable para la inducción de IL-17 e IL-23R. Otro factor que interviene en la diferenciación de las células Th17 es el receptor de hidrocarburos de arilo (AHR), el cual parece participar en la regulación de IL-22. Las células Th17 expresan altos niveles de IL-23R, e IL-1R. En humanos IL-1 es necesario para la diferenciación de las células Th17, aunque en modelos murinos e *in vitro* esto no es tan claro. IL-17A activa quinasas reguladas por señales extracelulares (ERK1 y ERK2), Jun y p38 en diversos tipos celulares [83]. IL-1 activa una gran variedad de vías proteicas como Myd88, IRAKs, TRAF6, NFκB y MAPK[84].

Th9

Las células Th9 están asociadas en la respuesta a gusanos parasíticos. Las células Th9 son necesarias para la respuesta contra parasitos como *Trichuris muris y N. brasiliensis*. Están asociadas con una serie de enfermedades autoinmunes como inflamación de las vías aeras y EAE[59, 60].

El principal producto de las células Th9 es IL-9, la cúal fue originalmente descrita como una citocina tipo Th2. IL-9 participa en la expulsión de parásitos intestinales, asma y leucemia[57]. PU.1 es el regulador maestro propuesto para las célula Th9. PU.1 esta asociado con el desarrollo de mieloide y de los linfocitos B, sin embargo aún hay muchas interrogantes sobre su regulación el los linfocitos T CD4+. La diferenciación de las células Th9 es altamente inestable y depende fuertemente del micro-entorno. IL-9 es inducido por PU.1, IRF4, y el TCR por medio de BATF y NFAT. TGFβinduce a IL-9 por medio de SMAD-3, TGFβesta involucrada en la diferenciación de Th17 y Treg. IL-2 induce a IL-9 por medio de STAT5, IL-2 esta involucrada con la activación de los linfocitos T CD4+ y la diferenciación de Th2[58, 59, 60].

Th22

Las células Th22 están asociadas a la respuesta inmune en la piel. Están asociadas con una serie de enfermedades autoinmunes como psoriasis, [61, 62].

El principales producto de estas células es IL-22, miembro de la familia de IL-10. La producción de IL-22 fue atribuido a Th17, aunque esta citocina también puede ser producida por células Th1 y Th2[61]. IL-22 esta asociada conde fase aguda y la respuesta inmune innata y enfermedades autoinmunes como enfermedad de Crohn, enfermedad intersticial del pulmon y artritis reumatoide[63]. No se conoce el regulador maestro de las célula Th22, aunque AHR esta fuertemente asociado con ellas. IL-22 puede ser activado en las células Th0 por TNF-α e IL-6. Las células Th22 expresan altos niveles de CCR4, CCR6, CCR10 y PDGFR.

Tfh

Las células Tfh están asociadas con la formación de centros germinales y maduración de afinidad de linfocitos B[64, 65]. Las células Tfh son necesarias para la respuesta mediada por linfocitos B y la formación de centros germinales[66]. Estas células también han sido asociadas con el control de la microbiota intestinal[85] y el éxito de las vacunas[67].

Los principales productos de las células Tfh son IL.21. IL-21 participa en la respuesta Th17. En los linfocitos Tfh la producción de IL-21 esta mediada por IRF4 y STAT3. IL-4 participa en la respuesta Th2 y en la maduración de los linfocitos B. En los linfocitos Tfh la producción de IL-4 esta mediada por NFAT y JunB. Cabe destacar que gran parte de la función de los linfocitos Tfh es mediada por receptores de membrana como CD40L, SAP, la familia SLAM, ICOS, PD-1,+ y BTLA, los cuales median su interacción con los linfocitos B. Las células Tfh expresan altos niveles del receptor CXCR5, e el cual esta asociado con la migración a folículos.

Bcl6 es el regulador maestro de las célula Treg. IL-21 induce la expresión de Bcl6 por medio de STAT3 y viceversa[86]. IL-21 también esta relacionada con las células Th17. Bcl6 es un represor transcripcional y es antagonista de Blimp1. Existen reportes contradictorios sobre si Bcl6 se autoinduce o se autoinhibe[66, 68]. IL-21 es capaz de autoinducirse por medio de STAT3. Sin embargo,cabe destacar que la diferenciación de las células Tfh depende fuertemente de su nicho y de sus interacciones con linfocitos B y APCs por medio de receptores de membrana.

Treg

Las células Treg median la auto-tolerancia. Estas células incluyen a las nTregs generadas en el timo y las iTreg inducidas en la periferia. Se puede distinguir a las iTreg por el marcaje epigenético en el locus de Foxp3 y los marcadores Dapl1 e Igfbp4[87, 88]. El incremento de células Treg en ratón previene el rechazo de implantes y combate enfermedades autoinmunes. La falta de células Treg está fuertemente relacionada con enfermedades autoinmunes, aunque también incrementa la inmunidad contra tumores e infecciones crónicas[89, 45].

Los principales productos de las células Treg son TGF-β, IL-10 e IL-35. TGF-β participa en la anergia, la tolerancia oral, la diferenciación de las células Treg y Th17. TGF-β regula múltiples procesos, las proteínas SMAD constituyen los componentes básicos de la cascada de señalización intracelular. Estudios recientes revelan que las proteínas SMAD y los receptores transmembranales de TGFβ están regulados [90]. IL-10 tiene funciones antiinflamatorias y anérgicas. Sin embargo no todas las células Treg producen IL-10 necesariamente. De la misma manera, IL-10 puede ser producido por células Th1, Th2 y Th17. Esto hace que sea posible que IL-10 sea una citocina de autoregulación propia de las Th. IL-35 comparte la subunidad ED13 con IL-12 e IL-27 y participa en la actividad supresora. Las Treg pueden además promover la anergia de las células Th a través del contacto célula-célula. Cabe destacar que una parte importante de las funciones de las Tregs son mediadas por receptores de membrana como LAP, que tiene función tolerigénica, y CD25, que media es secuestro de IL-2[89, 45].

Foxp3 es el regulador maestro de las célula Treg. TGF-β induce la expresión de Foxp3 y viceversa. El nivel de TGF-β necesario para inducir Foxp3 es mayor al necesario para inducir RORγt. STAT5 participa en la inducción de Foxp3 uniéndose a su promotor. TGF-β y Foxp3 no son necesarios ni suficientes para expresar IL-10. La mayoría de las Tregs expresan IL-2Rα (CD25) lo cual apunta a la importancia de IL-2. También expresan CTLA-4, GITR y Folr4 aunque no se conoce su función o relevancia[89, 45].

Tr1

Las células Tr1 están asociadas a la regulación de la inflamación y la tolerancia oral. Las células Tr1 son necesarias para prevenir respuestas inflamatorias excesivas y mantener la tolerancia inmune. Están asociadas con la supresión de enfermedades autoinmunes[71, 72].

Los principales productos de las células Tr1 son IL-10 y TGFβ. IL-10 es una citocina anti-inflamatoria que se produce en múltiples células del sistema inmune incluyendo otras células T CD4+. TGFβes una citocina que ha sido asociada tanto con la regulación al inducir a las células Treg, como la inflamación al ser necesaria por Th17[22]. No se conoce el regulador maestro de las célula Tr1, aunque se sabe que c-maf esta asociado con ellas. El estudio de las células Tr1 se ha visto complicado por la falta de marcadores claros. Las células Tr1 son Foxp3- y secretan bajos niveles de IL-2 y, algunas veces, IFNγ. La producción de IL-10 en células Tr1 puede ser inducida por IL-27, IL-15, IL-2 entre otras citocinas. IL-27 es una citocina de la familia de IL-27[71, 72, 22].

Th3

Las células Th3 están asociadas con la actividad anti-inflamatoria, y la tolerancia oral. Las células Th3 son necesarias para mantener la homeostasis en el intestino. Estas células son capaces de suprimir autoinmunidad e inflamación sistémica[73, 71].

Los principales productos de las células Th3 son TGFβy LAP. LAP es la forma latente de TGFβ, sin embargo, la presencia de una no garantiza la presencia de la otra. No se conoce el regulador maestro de las célula Th3, y su estudio de las células Tr1 se ha visto complicado por la falta de marcadores claros. Las células Th3 son Foxp3- y pueden expresar LAP y GARP. Estas células se generan en el intestino en respuesta a la presentación de antígenos orales en un entorno tolerigénico[73, 71].

1.2.2. Regulación cruzada

La diferenciación de células Th no solo está regulada por ciclos de autoinducción como los generados por IFNγ, IL-4, IL-21 o TGFβ. Los diferentes linajes también se inhiben entre ellos. Esto se logra a través de la represión por factores transcripcionales, por competencia sitio-específica en los receptores de citocinas por las proteínas SOCS y por factores epigenéticos.

Existen varias vías de inhibición dentro de las células Th [Fig: 1.5]. La regulación entre Th1 y Th2 es mediada por la competencia entre sus citocinas IFNγ e IL-4 y entre sus factores transcripcionales T-bet y GATA-3. T-bet inhibe la diferenciación en Th2 mediante la fosforilación de tirosinas de GATA3, impidiendo que éste se una al ADN[91]. T-bet y GATA3 también son inhibidos por Foxp3 y TGFβ. IFNγ e IL-4 se inhiben indirectamente a través de sus factores transcripcionales y de su activación de T-bet y GATA3. Curiosamente la competencia entre estas dos citocinas podría ser también directa, ya que se han reportado interacciones intercromosomales entre los genes de ambas citocinas[92].

La diferenciación de Treg y Th17 están también reguladas. La adición de IFNγ o IL-4 inhibe la expresión de RORγt, probablemente a través de T-bet y GATA3. TGFβ favorece la expresión de RORγt (Th17) y de Foxp3 (Treg) esto ha generado un interesante mecanismo de regulación. Foxp3 se une a RORα y RORγt e inhibe su activación en una manera dosis-dependiente. TGFβ lleva a la rápida inducción de RORγt, pero la unión de RORγt al promotor de IL-17 es suprimida

por su interacción con Foxp3. Al añadir IL-6 o IL-21 los niveles de Foxp3 disminuyen, impidiendo su acción inhibitoria. IL-6 no altera la expresión de RORγt, pero reprime a Foxp3 permitiendo la expresión de IL-17 [93]. TGFβ sólo en concentraciones de 1 ng/ml no induce a IL-17. Sin embargo, TGFβ a 10 ng/ml en combinación con IL-1, IL-21 o IL-23 promueve RORγt e IL-17. Mientras tanto 50 ng/ml suprimen a IL-17, probablemente a través de la activación de Foxp3[94].

La regulación de Th9, Th22 y Tfh esta menos estudiada. IL-9 es inhibido por IFNγ e IL-23. PU.1 inhibe la diferenciación hacia Th2, favoreciendo a Th9. IL-22 parece ser inhibido por c-maf e IL-10[63]. La diferenciación de Tfh esta regulada principalmente por su nicho. Sin embargo, se sabe que Bcl6 es inhibido por Blimp, el cual se encuentra sobre expresado en otras células T CD4+(31, 33). Foxp3 también inhibe a Bcl6[67]. Al mismo tiempo, Bcl6 es un inhibidor transcripcional, es cuál es capaz de inhibir a Tbet, GATA3 y RORγt[68].

IL-10 es una citocina anti-inflamatoria, está principalmente relacionada con las células Treg y Tr1, sin embargo puede ser producida por Th1, Th2 y Th17. IL-10 señaliza a través de STAT-3, al igual que IL-6, IL-21 e IL-23, lo cual es paradójico, dado que estas son citocinas pro-inflamatorias. Sin embargo, existen datos que señalan que la activación de STAT3 por IL-10 e IL-21 es diferente, ya que activan distintos programas transcripcionales. En las células Treg la expresión de Foxp3 y TGFβ no es necesaria ni suficiente para la expresión de IL-10. También es la citocina característica de las células Th3, sin embargo no se sabe si este es un verdadero linaje o es el producto de la expresión de IL-10 por Th1, Th2 o Th17[23]. Esto lleva a suponer que IL-10 es una citocina propia de todas las células Th que sirve como mecanismo de autoregulación.

Proteínas SOCS

Las proteínas supresoras de señalización de citocinas (SOCS) inhiben las vías de señalización de citocinas. Las citocinas activan las vías de señalización JAK-STAT de fosforilación al unirse a los receptores transmembranales. Las proteínas SOCS se unen al dominio de fosforilación impidiendo la activación de la vía JAK-STAT. La mayoría de estas proteínas SOCS son inducidas por citocinas y sirven como ciclos de autoinhibición y mecanismos de diferenciación celular. SOCS1 es inducida por STAT6 (IL-4) y por STAT1 (IFNγ), esta proteína se une a IL-4R, IFNαR, IFNγR y MyD88. SOCS1 también se une a IL-2 sobretodo en el timo durante la maduración de las células Th. SOCS3 es inducida por STAT6 (IL-4) y por IL-10, se une a IL-6R e IL-12R. SOCS5 es inducida por IL-12 y se une a IL-4R [5, 95, 4, 6] [Fig: 1.5].

1.2.3. Heterogeneidad

Las células T CD4+ pueden clasificarse en varios tipos celulares dependiendo del patrón de expresión de citocinas y factores transcripcionales maestros[43]. Sin embargo, también se han descrito células que expresan citocinas y factores transcripcionales asociados con otros tipos celulares[16, 25, 18, 96]. Al mismo tiempo, en un organismo pueden coexistir células T CD4+ con diferentes patrones de expresión[43]. De esta forma, las células T CD4+ son heterogéneas tanto en sus pa-

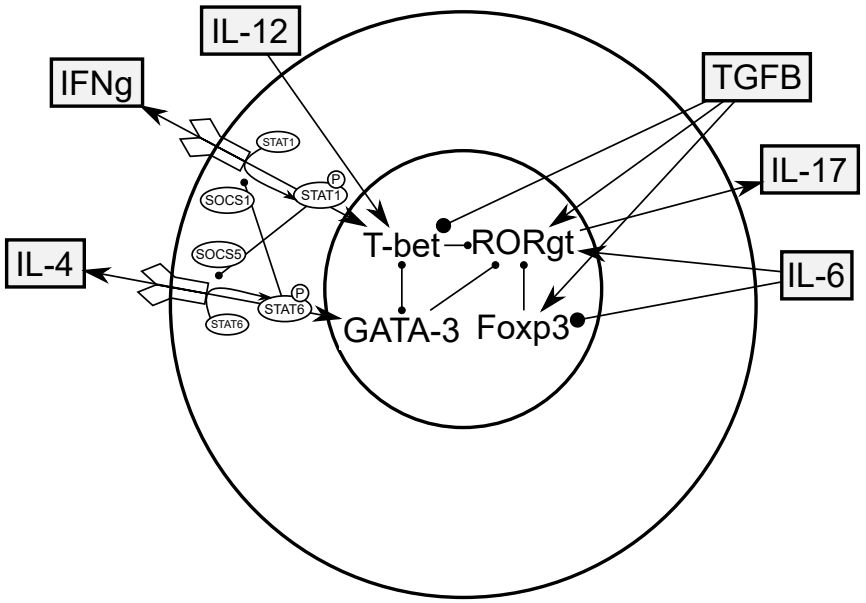


Figura 1.5: Regulación entre tipos celulares. La diferenciación de los linfocitos T CD4+ esta regulada a multiples niveles, incluyendo inhibiciones entre factores transcripcionales, y inhibición de las vías de señalización por proteínas SOCS.

trones de expresión como en su comportamiento poblacional. En este trabajo nos centraremos en la heterogeneidad de los patrones de expresión. Cabe destacar que la mayoría de los estudios de diferenciación de células Th son realizados *in vitro* y las células son estimuladas durante un largo tiempo. Es cuestionable si los mismos patrones de diferenciación que vemos *in vitro* son reproducibles *in vivo*, dado que las muestras *in vivo* muestran gran heterogeneidad y es difícil separar las células para determinar sus patrones de expresión de citocinas y factores transcripcionales.

Existen múltiples casos donde las células T CD4+ expresan factores transcripcionales o citocinas asociadas con otros subtipos. Incluso los tipos celulares que se consideran mutuamente excluyentes como Th1 y Th2 pueden presentar estados híbridos T-bet+GATA-3+ y producen tanto IFN-γcomo IL-4[97]. Se han reportado células Th17 que producen tanto IL-17 como IFNγ, sobretodo *in vivo*[22, 98]. Las células Th17 también pueden producir IL-22 junto con IL-17, aunque las células IL22+IL17- han sido caracterizadas como Th22[99, 100, 101]. Originalmente, IL-9 fue descrita como una citocina asociada con Th2 y el factor transcripcional PU.1 como parte de la hematopoiesis, aunque después ambos fueron asociados con Th9[102]. Las células Th9 también pueden producir IL-10 e IL- 21, aunque estas citocinas son reguladas independientemente de IL-9[103, 60]. Las células Tfh pueden expresar Bcl6 y CXCR5 junto con citocinas como IFN-γ, IL-4 o IL-17 en respuesta a entornos polarizantes hacia Th1, Th2 y Th17 respectivamente, mas aún estos patrones de expresión pueden ser también encontrados independientemente de estas vías de diferenciación[66, 104].

En las células reguladoras esta heterogeneidad de patrones e expresión esta asociada tanto con el control de la respuesta inmune como con la inflamación crónica. En las células Treg Foxp3+,

se han reportado células híbridas que colocalizan con las células efectoras. Estas células expresan Foxp3+ y sus marcadores como CD25+ junto con receptores, y factores transcripcionales asociados con Th1, Th2, y Tfh, por ejemplo, existen reportes de células Foxp3+Tbet+, Foxp3+GATA3+ y Foxp3+CXCR5[105, 18]. Estas poblaciones son fundamentales para suprimir la respuesta inmune de las células efectoras, ademas de que son capaces de expresar IL-10[106, 107, 105]. Existen además reportes de células que expresan tanto Foxp3 como citocinas proinflamatorias como IFNγo IL-17, las cuales se encuentran asociadas a inflamación crónica como la que se observa en pacientes obesos[108, 26, 109, 110]. También se han detectado células que expresan Foxp3+RORγt+, aunque esto último podría deberse a la acción competitiva entre estos dos factores [93]. Además, la expresión de los factores transcripcionales en las células Th no es fija, lo cual afecta su diferenciación, la expresión transitiva de Foxp3 se ha reportado[111, 112, 113]. La citocina IL-10 no solo esta asociada con Tr1, sino también es producida por células Th1, Th2, Th9, Th17 y Tfh[114, 115, 23, 116, 117, 24, 118, 119]. Esto hace sospechar que el programa de regulación de IL-10 es independiente de los factores transcripcionales maestros, resaltando el rol tolerigénico de esta citocina.

1.2.4. Plasticidad

Una vez diferenciadas las células T CD4+ pueden cambiar su patrón de expresión, mostrando plasticidad fenotípica. Se ha reportado que es posible transitar entre Th1 y Th2, sin embargo estas transiciones requieren entornos de citocinas particulares[120, 121, 17, 122]. Las células Th17 pueden expresar IFN-γe incluso expresan Tbet en respuesta a cambios en su entorno [123, 124, 125]. Además, las células Th17 pueden transitar hacia Treg y expresar Foxp3 e IL-10[126, 127, 128]. Las células Th9 surgen de células Th2 en presencia de TGFβ[102]. Las células IL-22 pueden diferenciarse a través de células Th17[129]. Las células Tfh pueden ser inducidas o transformarse en Th1, Th2, Th17 o Treg[130, 18, 66, 131]. Esta plasticidad es fundamental para la formación de centros germinales en el intestino[132, 133, 134].

Las células Treg pueden volverse Th17 en presencia de señales proinflamatirias como IL-1β, IL-6, IL-23[135]. Además, las células Treg pueden expresar un patrón de expresión similar a Th1, el cual ha sido asociado tanto con el control de la respuesta Th1 como con inflamación crónica[105, 18, 108, 26, 109, 110]. Varias señales entre las que destaca la citocina IL-27 pueden llevar a la producción de IL-10 por los distintos tipos celulares[136, 137, 138]. Esta transición plástica esta asociada con el control de la inflamación para evitar que esta dañe al organismo[139, 122, 136, 140, 128, 138, 141, 142]. Estas son solo algunas de las transiciones reportadas. La señalización por el TCR y cofactores, las proteínas SOCS, la vía PI3K–AKT–mTOR y el metabolismo también pueden afectar la plasticidad de las células T CD4+[18].

Recientemente, se han descrito diferentes formas de plasticidad entre los linajes de células Th [Fig: 1.6]. La transición entre células Treg y Th17 depende del entorno inflamatorio, dado que Foxp3 es inhibido por las citocinas inflamatorias. Es posible polarizar las células Th17 en células Th1 en presencia de IFNγ e IL-12. Se puede promover un estado híbrido Th1-2 al exponer a células Th2 a IFNγ e IL-12. Sin embargo, no se han observado algunas transiciones, como de Th2 a Th17 o

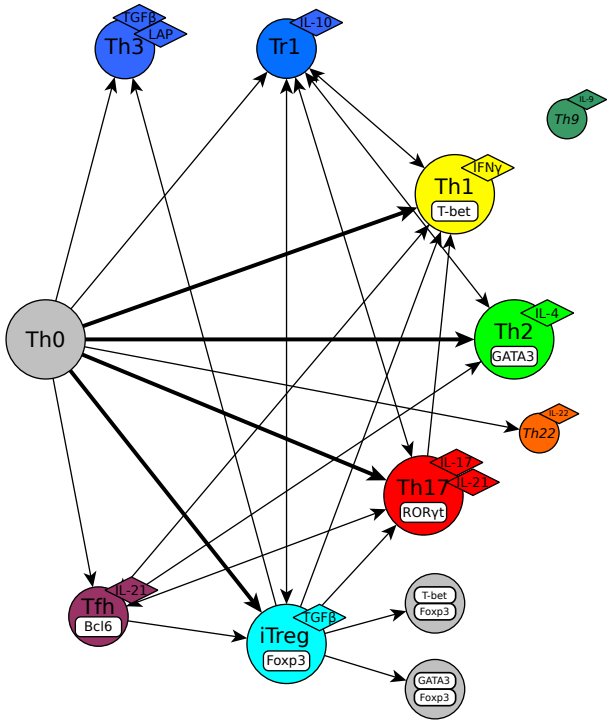


Figura 1.6: Plasticidad en células T CD4+. Una vez diferenciadas, las células TCD4+ pueden cambiar su patrón de expresión en respuesta a señales del entorno y otras perturbaciones.

Treg [17]. La estabilidad de las células es mantenida gracias a modificaciones epigenéticas y ciclos de autoinducción.

1.2.5. Procesos inflamatorios e hiperinsulinemia

Se ha visto que hay una correlación entre la obesidad y la inflamación crónica[Fig: 1.7]. El tejido visceral adiposo obeso tiene un contexto inflamatorio y tiene altas cantidades de citocinas como IL-6, IL-1 y TGFβ, macrófagos, células T citotóxicas, Th1 y Th17, mientras que los niveles de Treg e IL-10 se ven disminuidos [143, 27, 144]. La inflamación inicial podría ser causada por los mastocitos, ya que inhibir la activación de estos redujo el incremento de peso y la resistencia a la insulina en ratones [145].

Existe una retroalimentación entre la obesidad y la inflamación crónica. El exceso de ingesta de nutrientes, en particular azucares, incrementa la absorción de glucosa por los adipocitos y hepatocitos. Este proceso los somete a un estrés oxidativo, además de que estás células producen citocinas que favorecen el reclutamiento de macrófagos activados, células T citotoxicas, Th1 y Th17. Estás células producen citocinas proinflamatorias, las cuales favorecen la resistencia a la insulina de los adipocitos y hepatocitos[]. Esto afecta el metabolismo de la glucosa, favoreciendo indirectamente el

incremento de los niveles de insulina. Altos niveles de insulina inhiben la producción de la citocina IL-10 por las células T CD4+, afectando el balance entre células regulatorias e inflamatorias[29]. Además, los altos niveles de insulina favorecen la absorción de glucosa por los adipocitos y hepatocitos. De esta manera, la inflamación y la obesidad se retroalimentan mutuamente.

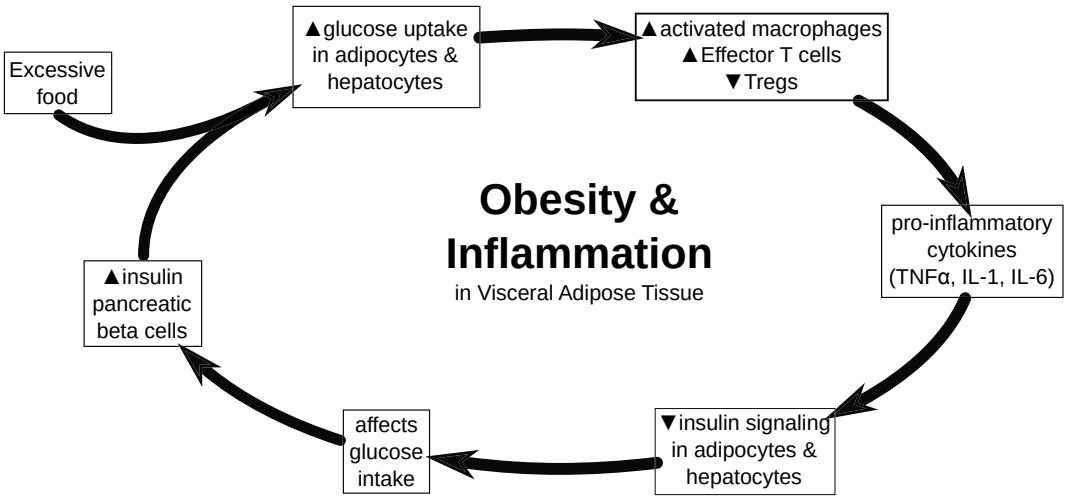


Figura 1.7: Inflamación crónica asociada a obesidad. Existe una retroalimentación entre la obesidad y la inflamación crónica.

1.3. Redes Booleanas

La mayoría de los sistemas complejos de la biología, como la regulación genética, el sistema nervioso, el sistema inmune o los ecosistemas, están compuestos de múltiples elementos que interactúan entre si. Estos sistemas pueden ser modelados con redes. Una red es una colección de elementos conectados. Puede ser visualizada como un conjunto de nodos que corresponden a los elementos de la red y un conjunto de aristas que representan las interacciones entre los elementos. Cada nodo tiene un valor de salida y varios valores de entrada, además de una regla que define el valor de la salida en función de las entradas. Las salidas y las entradas están definidas por las interacciones con otros elementos de la red.

Los sistemas biológicos pueden ser modelados como redes. Los nodos representan elementos como genes, proteínas, células u organismos. Mientras tanto las interacciones entre ellos corresponden a las aristas. Sin embargo, dada la cantidad de elementos y la complejidad de las interacciones en los sistemas biológicos es difícil generar una descripción exacta del sistema. Es por esto que se recurre a simplificaciones como suponer tiempos discretos o que los elementos sólo pueden tener un número limitado de valores. Una de estas simplificaciones son las redes Booleanas.

En una red de regulación Booleana cada nodo representa un gen o proteína. El nivel de activación del nodo es la cantidad de proteína correspondiente al gen que ha sido sintetizada. En muchos sistemas biológicos el nivel de expresión de un gen se mantiene en un nivel basal constitutivamente. Cuando los factores activadores sobrepasan un umbral, entonces la cantidad de la proteína correspondiente se incrementa rápidamente hasta alcanzar un máximo. Esto permite suponer que la expresión del gen puede ser expresada en base a funciones Booleanas, con el gen inactivo (expresión basal) o activo (incremento de la función).

Las aristas de una red de regulación Booleana representan la forma en que otros genes ayudan a la activación o represión de la transcripción del gen. Una arista de activación que une a dos nodos implica que uno de los nodos es un factor transcripcional del otro, fomentando la síntesis de la proteína correspondiente. Por otro lado, si la arista representa una inhibición entonces uno de los genes es un inhibidor de otro, evitando que el gen blanco sea sintetizado.

En 1969 Kauffman propuso a las redes booleanas como modelos de regulación genética [31]. Partió de la idea de que un gen puede ser expresado (1) o no expresado (0). Dado que la expresión de cada gen es controlada por la expresión de otros genes Kauffman supuso que el genoma es una red donde una arista del gen A al gen B representa que A regula la expresión de B ya sea activándolo o inhibiéndolo. Dado que no existían suficientes datos experimentales Kauffman generó redes aleatorias. Sin embargo, su generalidad ha motivado interés más allá de su propósito original [146, 147].

Las redes booleanas son un tipo de red dinámica discreta, finita y determinista. Una red booleana consiste de N nodos unidos por K conexiones cada uno. Cada nodo tiene k aristas de entrada y l aristas de salida. Los nodos son booleanos, es decir su estado es "prendido" (1) o "apagado" (0). El estado de cada nodo se actualiza en un tiempo discreto, el estado en el tiempo $t+1$ depende de los estados de sus K entradas en el tiempo t a través de una función booleana [Fig: 1.8].

Según Kauffman, los estados estacionarios de las redes de regulación pueden representar diferentes tipos celulares dependiendo del estado de los nodos o genes. Dada una configuración inicial y las relaciones entre los genes, se puede dejar evolucionar el sistema y encontrar que los elementos de la red tienen estados de activación cíclicos o de punto fijo, que corresponden a los atractores. Estos estados estacionarios o atractores, no son estados intrínsecos de cada elemento, sino un fenómeno emergente de la complejidad del sistema. En base a los datos experimentales disponibles se han podido inferir redes de regulación de varios organismo como *E. coli, S. cerevisae, B. subtilis* [148], la morfogénesis de las flores de *A. thaliana*[149], los genes de polaridad de segmentación de *D. meganogaster* [150] y la diferenciación de células Th [151, 152, 153].

1.3.1. Funciones booleanas

La salida de un nodo está dada por una función booleana que toma como argumentos sus entradas. Si un nodo tiene N entradas estas pueden estar en 2^N estados diferentes. Cada regla le asigna una sola salida a cada posible estado de las entradas. Sin embargo, todas estas funciones se pueden expresar en base a tres funciones booleanas: *and*, *or* y *not* [Cuadro: 1.1 y 1.2].

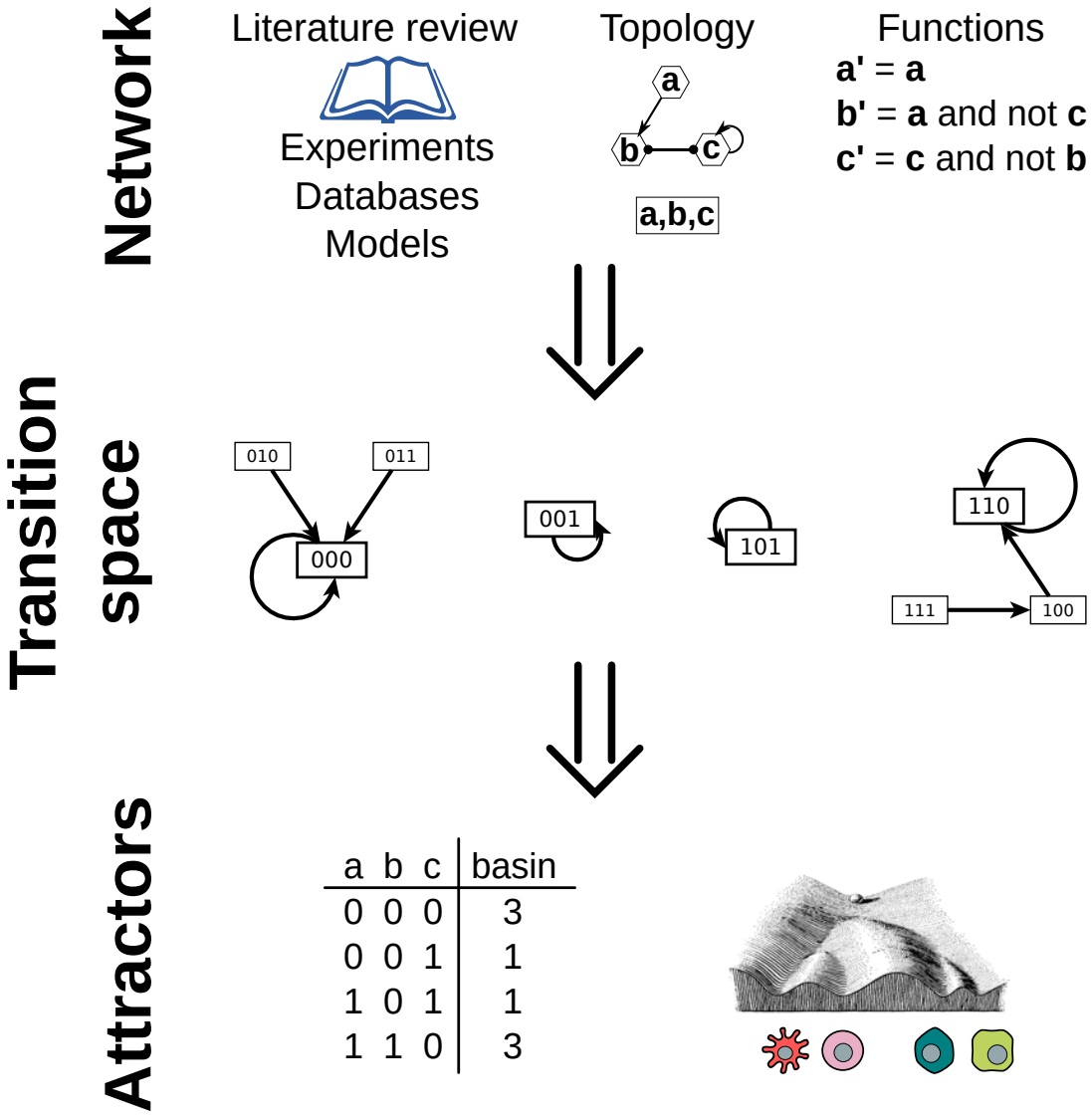


Figura 1.8: Redes Booleanas. Las redes Booleanas se obtienen a partir de los datos reportados, y constan de nodos, las interacciones entre ellos, y las funciones que recapitulan la regulación de los nodos. El valor de los nodos es actualizado hasta que se llega a un estado estable o a un ciclo. Las soluciones de la red corresponden a los tipos celulares.

No siempre se conocen las funciones booleanas de una red. Cuando es así se pueden inferir las funciones identificando los elementos del sistema, sus relaciones y tipo de interacciones. La formulación y análisis de redes permite encontrar nuevo conocimiento y la posibilidad de hacer predicciones. Un grafo puede tener varias funciones booleanas diferentes; al inferir sus reglas es necesario buscar aquellas que resultan más parsimoniosas y consistentes con las observaciones experimentales.

t		$t+1$	
A	B	A *and* B	A *or* B
0	0	0	0
0	1	0	1
1	0	0	1
1	1	1	1

Cuadro 1.1: Funciones and y or

t	$t+1$
A	*not* A
0	1
1	0

Cuadro 1.2: Función not

En el caso de los sistemas biológicos existen varias formas de inferir las funciones de una red de regulación. Se puede utilizar la información ya conocida de la expresión de genes y proteínas, incluyendo la regulación transcripcional, postranscripcional, traduccional y postraduccional. También se pueden realizar inferencias de la función de una proteína analizando las diferencias en las respuestas a diversos estímulos en organismos silvestres y mutantes. Una vez propuesta la relación de regulación entre dos genes, ésta puede ser confirmada experimentalmente.

1.3.2. Espacio de transiciones

Las redes booleanas son deterministas y finitas. Una red de N nodos tiene 2^N estados posibles. Cada estado corresponde a un nodo de la red de estados de la red original. Las aristas de esta red se construyen tomando uno de los estados, evaluándolo de acuerdo a las funciones de la red y viendo cual es el nuevo estado obtenido. De esta forma, en una red booleana determinista, cada estado tiene una arista de salida que lleva a su sucesor.

El estado de un nodo X en el tiempo t es una función Booleana de sus N reguladores en el tiempo anterior tal que: $X_{t+1} = f(A_t, C_t, ..., N_t)$

A partir del estado inicial X_0 en el tiempo $t = 0$ se pueden aplicar las funciones Booleanas para determinar la trayectoria que seguirá el sistema. Las funciones pueden ser evaluadas sícronamente evaluando todos los nodos al mismo tiempo, o asincronamente evaluando los nodos uno por uno. En el caso de la actualización síncrona cada estado tiene un solo sucesor determinista, en el caso de la actualización asíncrona un estado puede tener varios estados sucesores, y cual se elija dependerá del orden en que los nodos sean evaluados[154]. Eventualmente, al ser los estados posibles finitos, el sistema regresara a un estado ya visitado.

1.3.3. Atractores

Cuando un estado es revisitado la red ha alcanzado una solución o atractor. El número de estados que tarda en reencontrarse un estado específico determina el periodo de la solución. Las soluciones puntuales tienen un periodo de uno (un solo estado), mientras que los cíclicos tienen periodos más grandes que uno (estados múltiples). Una red booleana puede tener uno o más atractores.

El conjunto de estados visitados hasta que un atractor es alcanzado se llama transitorio. El conjunto de estados llevando hacia un atractor forma su cuenca de atracción. La cuenca de los

diferentes atractores divide el espacio de estados [Fig: 1.8]. Los estados sin un predecesor se llaman cúspides, dado que sólo pueden ser alcanzados si son la condición inicial y en la siguiente iteracción se pierden.

Es importante notar la diferencia entre la topología de una red booleana y su red de estados. En los sistemas biológicos, la red booleana representa las interacciones entre los genes y proteínas del sistema, es decir como los N nodos de la red se afectan unos a los otros. En contraste, la red de estados representa las posibles combinaciones de activación de genes y proteínas, donde cada estado estacionario corresponde a un estado de diferenciación de la célula. En la red de estados cada nodo representa un estado de la red booleana, donde hay 2^N estados, y la red representa las transiciones de todo el espacio de estados. Las soluciones a las cuales converge el sistema representan los tipos celulares. Uno de los principales temas en el estudio de las redes booleanas es entender como cambios en la topología de la red (escala baja) afectan la red de estados (escala alta), lo cual no es trivial.

1.3.4. Modelos previos

Estudios anteriores han utilizado redes de regulación Booleanas para estudiar la diferenciación y la plasticidad de los linfocitos T CD4+[152, 153, 155, 156, 157, 35, 158]. Estos modelos han logrado capturar la regulación dinámica y no lineal de las células T CD4 + y recuperaron los atractores correspondientes a los tipos de células Th0, Th1, Th2, iTreg y Th17[152] y su comportamiento poblacional[155]. Estos modelos también han sido usados para estudiar la linfopoyesis de las células T CD4+, T CD8+ y B[157, 158]. Usando estos modelos se han realizado estudios preliminares de la plasticidad de las células T CD4+ en presencia de diferentes citocinas en el microambiente[153] y el efecto de una molécula específica (PPARγ) en la transición Th17 / iTreg[156]. Además, estos modelos han sido capaces de recuperar la diferenciación de células Th9 y Th22 y predecir que transiciones entre tipos celulares son posibles[35]. Sin embargo, estos modelos no explican la diferenciación de tipos celulares como Tr1 o Th3. Además, aún hace falta un estudio sistemático de los efectos de los diferentes entornos biológicos y determinar que nodos son fundamentales para la plasticidad entre tipos celulares.

Capítulo 2

A Minimal Regulatory Network of Extrinsic and Intrinsic Factors Recovers Observed Patterns of CD4+ T Cell Differentiation and Plasticity

PLOS | COMPUTATIONAL BIOLOGY

RESEARCH ARTICLE

A Minimal Regulatory Network of Extrinsic and Intrinsic Factors Recovers Observed Patterns of CD4+ T Cell Differentiation and Plasticity

Mariana Esther Martinez-Sanchez[1,2], Luis Mendoza[3], Carlos Villarreal[2,4], Elena R. Alvarez-Buylla[1,2]*

1 Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, Coyoacán, México Distrito Federal, México, **2** Centro de Ciencias de la Complejidad, Universidad Nacional Autónoma de México, Coyoacán, México Distrito Federal, México, **3** Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México Distrito Federal, México, **4** Departamento de Física Teórica, Instituto de Física, Universidad Nacional Autónoma de México, México Distrito Federal, México

* eabuylla@gmail.com

Citation: Martinez-Sanchez ME, Mendoza L, Villarreal C, Alvarez-Buylla ER (2015) A Minimal Regulatory Network of Extrinsic and Intrinsic Factors Recovers Observed Patterns of CD4+ T Cell Differentiation and Plasticity. PLoS Comput Biol 11(6): e1004324. doi:10.1371/journal.pcbi.1004324

Editor: Josep Bassaganya-Riera, Virginia Tech, UNITED STATES

Received: December 5, 2014

Accepted: May 7, 2015

Published: June 19, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Additionally, the models presented can be found at BioModels Database (acession numbers: MODEL1411170000 and MODEL1411170001). URL: https://www.ebi.ac.uk/biomodels/reviews/MODEL1411170000-1/

Funding: This work was supported by grants from: Consejo Nacional de Ciencia y Tecnología México (www.conacyt.mx): 180380 to CV and ERAB, and 180098 to (ERAB). Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica

Abstract

CD4+ T cells orchestrate the adaptive immune response in vertebrates. While both experimental and modeling work has been conducted to understand the molecular genetic mechanisms involved in CD4+ T cell responses and fate attainment, the dynamic role of intrinsic (produced by CD4+ T lymphocytes) versus extrinsic (produced by other cells) components remains unclear, and the mechanistic and dynamic understanding of the plastic responses of these cells remains incomplete. In this work, we studied a regulatory network for the core transcription factors involved in CD4+ T cell-fate attainment. We first show that this core is not sufficient to recover common CD4+ T phenotypes. We thus postulate a minimal Boolean regulatory network model derived from a larger and more comprehensive network that is based on experimental data. The minimal network integrates transcriptional regulation, signaling pathways and the micro-environment. This network model recovers reported configurations of most of the characterized cell types (Th0, Th1, Th2, Th17, Tfh, Th9, iTreg, and Foxp3-independent T regulatory cells). This transcriptional-signaling regulatory network is robust and recovers mutant configurations that have been reported experimentally. Additionally, this model recovers many of the plasticity patterns documented for different T CD4 + cell types, as summarized in a cell-fate map. We tested the effects of various micro-environments and transient perturbations on such transitions among CD4+ T cell types. Interestingly, most cell-fate transitions were induced by transient activations, with the opposite behavior associated with transient inhibitions. Finally, we used a novel methodology was used to establish that T-bet, TGF-β and suppressors of cytokine signaling proteins are keys to recovering observed CD4+ T cell plastic responses. In conclusion, the observed CD4+ T cell-types and transition patterns emerge from the feedback between the intrinsic

Universidad Nacional Autónoma de México (dgapa.unam.mx/html/papiit/papit.html): IN203113; IN204011; IN226510-3 and IN203814 to ERAB. Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica Universidad Nacional Autónoma de México (dgapa.unam.mx/html/papiit/papit.html): IN200514 to LM. MEMS acknowledges support from the graduate program "Doctorado en Ciencias Biomédicas, de la Universidad Nacional Autónoma de México". The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

or intracellular regulatory core and the micro-environment. We discuss the broader use of this approach for other plastic systems and possible therapeutic interventions.

Author Summary

CD4+ T cells orchestrate adaptive immune responses in vertebrates. These cells differentiate into several types depending on environmental signals and immunological challenges. Once these cells are committed to a particular fate, they can switch to different cell types, thus exhibiting plasticity that enables the immune system to dynamically adapt to novel challenges. We integrated available experimental data into a large network that was formally reduced to a minimal regulatory module with a sufficient set of components and interactions to recover most CD4+ T cell types and reported plasticity patterns in response to various micro-environments and transient perturbations. We formally demonstrate that transcriptional regulatory interactions are not sufficient to recover CD4+ T cell types and thus propose a minimal network that induces most observed phenotypes. This model is robust and was validated with mutant CD4+ T phenotypes. The model was also used to identify key components for cell differentiation and plasticity under varying immunogenic conditions. The model presented here may be a useful framework to study other plastic systems and guide therapeutic approaches to immune system modulation.

Introduction

The immune system protects organisms against external agents that may cause various types of diseases. As the immune system mounts specialized responses to diverse pathogens, it relies on plastic responses to changing immunological challenges. At the same time, the immune system must maintain homeostasis and avoid auto-immune responses. Therefore, the immune system relies on resilience mechanisms that enable it to return to basal conditions once pathogens or immunogenic factors are no longer present [1–3].

CD4+ T cells, also known as T helper (Th) cells, are key in the response to infectious agents and in the plasticity of the immune system. Naive CD4+ T cells (Th0) are activated when they recognize an antigen in a secondary lymphoid organ. Depending on the cytokine milieu and other signals in their micro-environment, CD4+ T cells attain different cell fates [2,4–7]. Nonetheless, we still do not have a complete understanding of the dynamic mechanisms underlying CD4+ T cell differentiation and plasticity [5].

Each CD4+ T cell type is associated with specific cytokines, receptors, transcription factors and functions (Fig 1). Th1 cells express T-bet, secrete interferon-γ (IFN-γ) and are associated with cellular immunity [8]. Th2 cells express GATA3, secrete interleukin (IL)-4 and are associated with immunity to parasites [8]. Th17 cells express RORα and RORγt, secrete IL-17 and IL-21, and are associated with neutrophil activation [9–10]. Follicular helper CD4+ T cells (Tfh) express Bcl6 and CXCR5, secrete IL-21 and are associated with B cell maturation in germinal centers [11,12]. Th9 cells secrete IL-9 and exert anticancer activity [13,14]. Induced regulatory T cells express Foxp3, secrete TGF-β and/or IL-10, and are associated with immune tolerance [15,16]. There is also considerable overlap among the expression profiles of different CD4+ T cells. For example, IL-9 and IL-10 can be secreted by Th1, Th2, Th17, iTreg cells and a variety of other immune cells [17–19]. T regulatory cells can also express IL-17 [20].

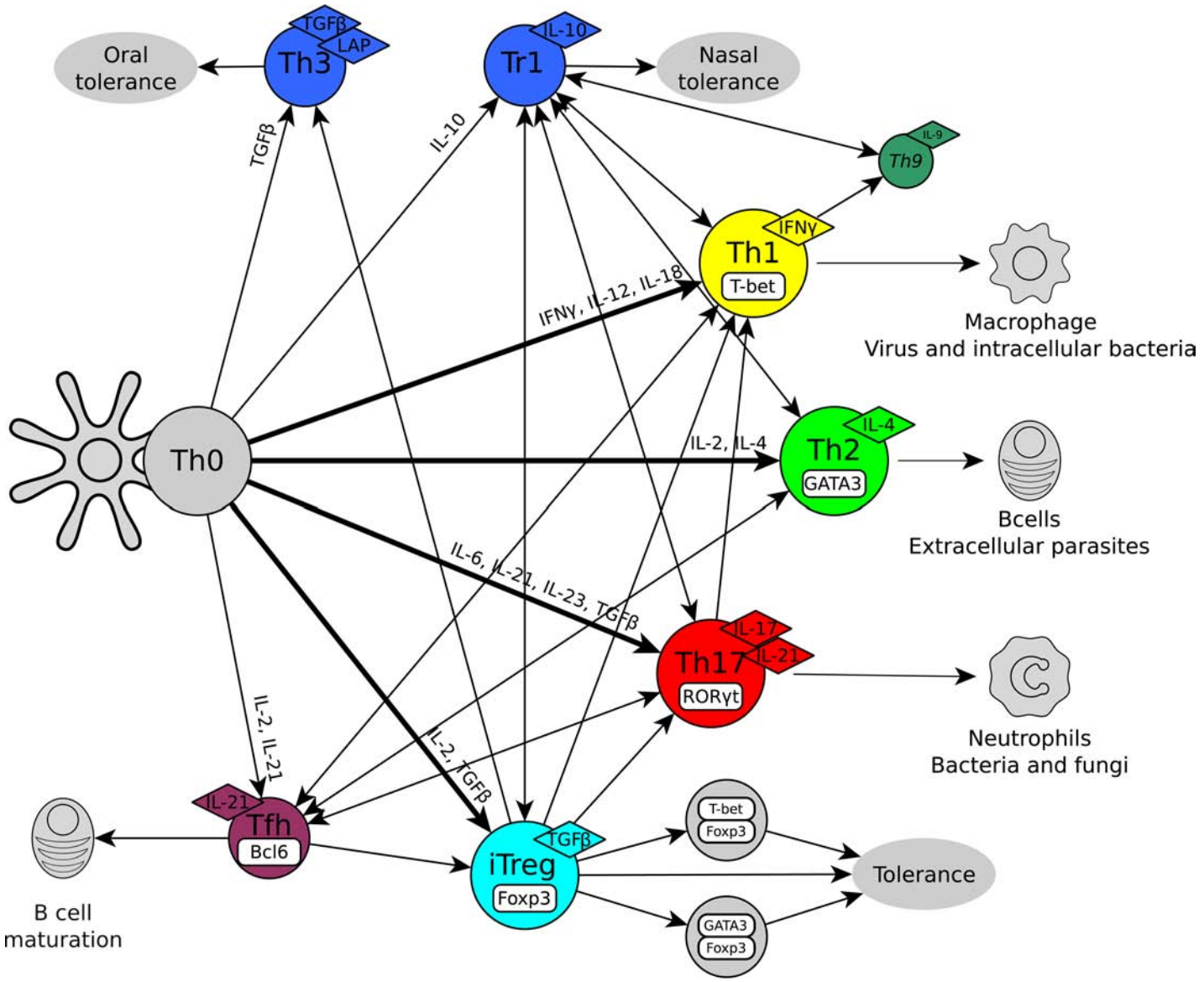


Fig 1. Differentiation and plasticity of CD4+ T cell types. CD4+ T cell types are characterized by their unique cytokine production profiles, transcription factors and biological functions. The main cell types are Th0, Th1, Th2, Th17, iTreg and Tfh. Other possible cell types have been described such as IL-9 (Th9), IL-10+Foxp3-(Tr1) and TGF-β+Foxp3-(Th3) producing cells.

doi:10.1371/journal.pcbi.1004324.g001

CD4+ T cells are highly plastic, switching from one type to another in response to environmental challenges (Fig 1) [1,21–23]. Th17 cells can transform into Th1 cells [24–25], and iTregs differentiate into Th17 in the presence of IL-6 [26]. Th2 cells can become IL-9 producing cells but may not easily become Th1 cells [27]. iTreg and Tfh cells can independently develop into other CD4+ T cell types, and they can be derived from Th1, Th2 or Th17 cells [28–30]. The differentiation and plasticity of CD4+ T cells depends on the interactions among the cytokines produced by other immune cells, epithelial cells, adipocytes, or by the CD4+ T cells themselves; the transduction of those signals and the regulation of this signaling by suppressors of cytokine signalling (SOCS) proteins; the set of transcription factors expressed inside the cells; epigenetic regulation; certain metabolites; and also microRNAs [4,6,31–33]. Given the

complexity of CD4+ T cell transitions and the difficulty of classifying a particular expression pattern as a subset or a lineage, we will refer to the different stable expression patterns of CD4 + T cells as "cell-types".

A mechanistic, integrative and system-level understanding of CD4+ T cell differentiation and plasticity requires dynamic regulatory network models that consider the concerted action of many components. These models can be used to prove whether the known biological interactions are necessary and sufficient to recover attractors that correspond to experimentally observed configurations in different CD4+ T cell types. Additionally, such models may be used to address whether the considered components and interactions also restrict and explain the observed patterns of transition among cell types. Finally, this type of model can be used to test the role of different network components in cell differentiation and plasticity.

In such regulatory network models, the nodes correspond to the regulatory components of the network such as genes, proteins or signals, while the links correspond to the interactions among components. The state of each node is determined by the expression level of its regulators, and the logical functions describe the dynamic evolution of the node states. The attractors, the states to which such regulatory networks converge, can be interpreted as the profiles characterizing different cell types (see reviews in: [34–36]).

Previous studies have used regulatory network models to study CD4+ T cell differentiation and plasticity [37–40]. These models captured the dynamic and non-linear regulation of CD4 + T cells and recovered the attractors corresponding to the Th0, Th1, Th2, iTreg and Th17 cell types. They have also been useful for preliminary studies of CD4+ T cell plasticity in the presence of different cytokines in the micro-environment [38] and fir studies of the effect of a specific molecule (PPARγ) in the Th17/iTreg switch [40]. However, as new T CD4+ cell types such as Tfh, regulatory Foxp3-independent, Th9, and Th22 cells are described, it is necessary to develop an updated regulatory network that is able to recover the configurations that characterize such novel cell subsets. Additionally, to date no minimal model that incorporates the necessary and sufficient set of interactions to also recover the reported patterns of transitions among Th cells has been reported.

Here, we specifically address whether CD4+ cell types and their transition patterns emerge as a result of the feedback between a minimal regulatory core of intra-cellular transcription factors and cytokines produced by the CD4+ T cell together with cytokines produced by other cells present in the micro-environment. Our results confirm that a regulatory network model that only considers the interactions among the master transcription factors is not sufficient to recover configurations that characterize the different CD4+ T cell types. Therefore, we then integrated a minimal network of master transcriptional factors with cytokine signaling pathway, including the cytokines produced by the cell and those present in the micro-environment, to integrate a network with the necessary and sufficient set of components to recover documented CD4+ T cell differentiation and plasticity patterns. The observed configurations of CD4+ T cells (Th0, Th1, Th2, Th17, iTreg, Tfh, Th9 and Foxp3-independent T regulatory cells) emerge from the feedback and cooperative dynamics among the multiple levels of regulation considered in the minimal model. In addition, this system is able to recover the plastic transition patterns and stability behavior that have been described for the different cell types in response to transitory perturbations and different micro-environments. Interestingly, our model predicts that transitions from particular cell types to others are caused by transient activations, while transient inhibitions usually cause cells to remain in their original cell types. Additionally, we show that T-bet, TGF-β and SOCS proteins are keys to recovering observed CD4+ T cell plastic responses. Finally, we discuss the relevance of our models for a system-level understanding of mammalian immunological responses and eventual biomedical interventions.

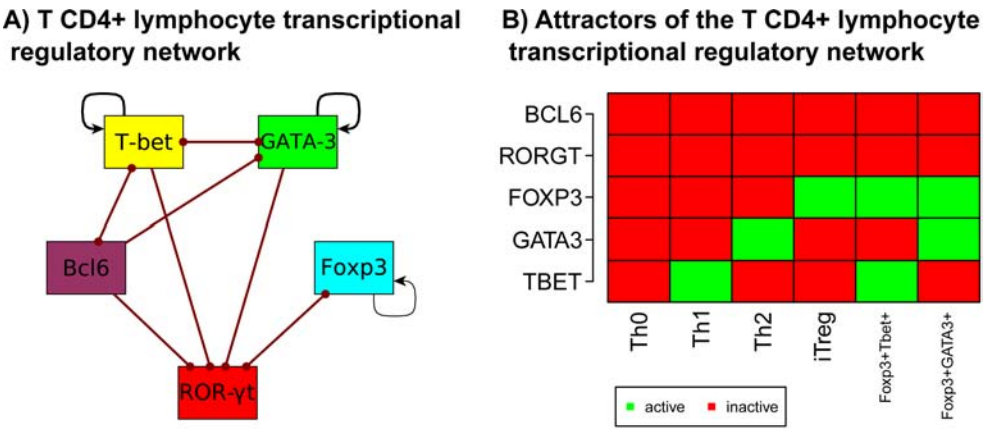


Fig 2. Minimal network of master transcriptional regulators CD4+ T (CD4+ T TRN). Based on published experimental data we constructed a CD4+ T cell regulatory network that includes the master transcriptional regulators and the interactions among those regulators (CD4+ T TRN). **(A)** Graph of the CD4+ T TRN. Node colors correspond to cell types: Th1 (yellow), Th2 (green), Th17 (red), iTreg (blue) and Tfh (purple). Activations among elements are represented with black arrows and inhibitions with red dotted arrows. **(B)** Attractors of the CD4+ T TRN: Each column corresponds to an attractor. Each node can be active (green) or inactive (red). The attractors correspond to configurations that characterize the Th0, Th1, Th2, iTreg, T-bet +Foxp3+ and GATA3+Foxp3+ types. The attractors corresponding to the Th17 and Tfh types could not be recovered.

doi:10.1371/journal.pcbi.1004324.g002

Results

CD4+ T cell regulatory network

Boolean networks are capable of integrating qualitative interactions (molecular, physical, chemical, etc.) into a coherent picture and are useful ways to explore the minimal set of restrictions that are necessary and sufficient to produce emergent biological patterns and behaviors [41–43]. The regulatory interactions considered in the present model are grounded on experimental data. In the proposed regulatory network, the nodes represent the regulatory components of the network and the links the interactions among them (S1 Table and S1 Fig). Given the complexity of the network, we simplified the model by removing intermediate components along a network path (S1 File) following a method proposed in [44] and checked the consistency of the reduced network using GINsim [45].

The predicted cell phenotypes arising from the steady states of the network are consistent with the available experimental data [2,4–7]. The model assumes that all interactions are synchronous, that all cytokine receptors are present, and that the TCR and its cofactors are activated (being unable to model unactivated and anergic CD4+ T cells). The model ignores weak interactions, low levels of expression, and epigenetic regulation (S1 File).

A core of master transcriptional regulators is not sufficient to explain CD4+ T cell differentiation

To address whether a minimal transcriptional regulatory core could recover the observed configurations that characterize the main CD4+ T cell types that have been described up to now, we extracted from the general network under study a minimal regulatory module consisting only of transcriptional regulators (Fig 2A, S2 Table, BioModels Database: MODEL1411170000). Our aim was to test whether this minimal module contained a sufficient set of interactions to predict the observed configurations for the transcription factors included in the model that characterize different CD4+ T cell types. The nodes of the transcriptional

regulatory network (TRN) correspond to the five "master" transcription factors associated with CD4+ T cell types: T-bet for Th1, GATA3 for Th2, RORγt for Th17, Foxp3 for iTreg, and Bcl6 for Tfh.

The dynamic analysis of this TRN recovered attractors corresponding to different CD4+ T cell types (Fig 2B): Th0, Th1, Th2, iTreg and the hybrid states T-bet+Foxp3+ [46] and GATA3 +Foxp3+ [47]. However, this TRN did not converge to configurations that characterize the Th17 and Tfh cell types, implying that the expression of RORγt and Bcl6 is not sufficient to maintain such cell types. This result may be caused by the lack of feed-forward loops in the TRN. RORγt has no positive interactions with any of the transcription factors considered in the TRN and lacks a feedback loop mediated by transcription factors [48]. The mode of self-regulation of Bcl6 remains unclear, as it has been reported to either activate or inhibit its own expression in B cells [49–50].

CD4+ T cell differentiation patterns emerge from feedback between the transcriptional regulatory network, cytokines and signaling pathways

The above result reveals which T CD4+ cell types rely only on the postulated TRN and which require extrinsic signals. To formally test this hypothesis, we extended the TRN network by introducing key components of signaling pathways and their regulators, as well as cytokines that have been shown to be fundamental in CD4+ T cell type attainment. This T CD4+ cell transcriptional-signaling regulatory network (TSRN) was then simplified (S1 File, S1 Fig) to obtain a minimal network. To reduce the number of nodes in the network, we assumed that the TCR signal was present and that the cytokine receptors were present in sufficient amounts to transduce a signal. This network lacks many important inflammatory cytokines (such as IL-1, TNFα), because while these cytokines are crucial for the immune response, they are dispensable for CD4+ T cell differentiation. The model analyzed in this paper also lacks extrinsic cytokines produced by other immune system cells and other cell types such as IL-12 and IL-18. The network also lacks some transcription factors and cytokines associated with newly reported Th types such as IL-22, as detailed experimental information linking them to the network model under analysis is not yet available.

The nodes of the simplified TSRN represent (Fig 3A, S3 Table, BioModels Database: MODEL1411170001) transcription factors, signaling pathways and extrinsic cytokines. The nodes corresponding to cytokine pathways are active if the signal is transduced; this means that if the cytokine is present, it forms a complex with the receptor that can activate a messenger molecule (for example a STAT protein), which is then translocated to the nucleus. Cytokines can be produced by both CD4+ T cells (intrinsic) and by other cells of the immune system and the organism (extrinsic). To resolve this ambiguity we added nodes representing the extrinsic cytokines produced by other cells and tissues of the immune system (IL_e). This extended TSRN includes 18 nodes: the transcription factors (Tbet, GATA3, RORγt, Foxp3, Bcl6), the effector cytokines and their signaling pathways (IFN-γ, IL-2, IL-4, IL-21, IL-9), the regulatory cytokines (TGF-β and IL-10) and the extrinsic cytokines (IFN-γe, IL-2e, IL-4e, IL-21e, TGF-βe and IL-10e). While IL-10, IL-6 and IL-21 all signal using STAT3, IL-6 and IL-21 cause inflammation, while IL-10 suppresses inflammation. To analyze this network, we assume that IL-10 signaling was mediated by a different pathway than IL-6/IL-21, even though they share STAT3 as a messenger molecule. The production of these external cytokines is independent of regulation inside the CD4+ T cell, but their signaling can be blocked (for example by SOCS proteins [51]). The resulting network includes two levels of regulation, the regulation in the nucleus by mutually inhibiting transcription factors and the regulation among the receptors and their signal transduction pathways mediated by SOCS proteins.

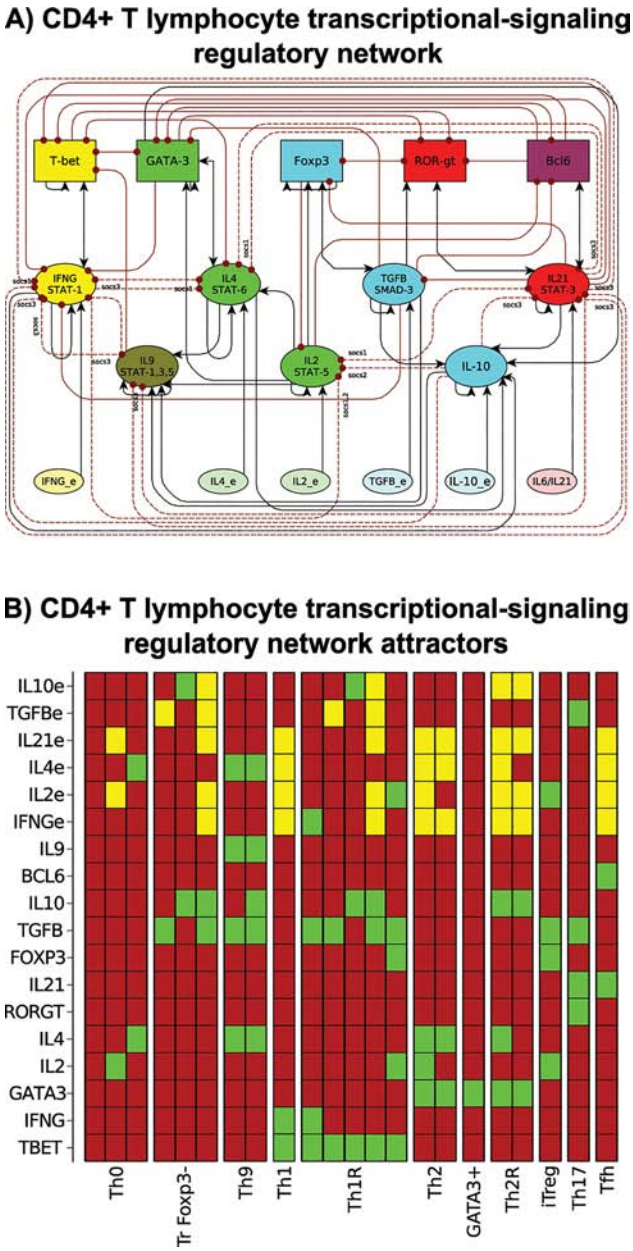


Fig 3. CD4+ T cell transcriptional-signaling regulatory network (TSRN). We constructed a regulatory network using available experimental data. The network includes transcription factors, signaling pathways, and intrinsic and extrinsic cytokines. **(A)** Graph of the TSRN. The nodes include transcription factors (rectangles), intrinsic cytokines and their signaling pathways (ellipses) and extrinsic cytokines (ellipses). Node colors correspond to cell type: Th1 (yellow), Th2 (green), Th17 (red), iTreg (blue), Tfh (purple), and Th9 (brown). Activations between elements are represented with black arrows, and inhibitions with red dotted arrows. The dotted lines represent inhibition mediated by SOCS proteins. **(B)** Attractors of the TSRN. Each column corresponds to an attractor. Each node can be active (green) or inactive (red), extrinsic cytokines may be active or inactive (yellow). The following attractors were found in the network: Th0, Th1, Th2, Th17, iTreg, Tfh, Th9 producing T cells, Foxp3-independent T regulatory cells (TrFoxp3-), T-bet+ T regulatory cells (Th1R), GATA3+ T regulatory cells (Th2R) and GATA3+IL-4- cells. Attractors where labeled according to the active transcription factors and intrinsic cytokines.

doi:10.1371/journal.pcbi.1004324.g003

The dynamic analysis of the TSRN yields stable configurations that correspond to: Th0, Th1, Th2, Th17, iTreg, Tfh, T regulatory Foxp3-independent cells, Th1R, Th2R and GATA3 +IL4- cells (Fig 3B). As this biological patterns can be obtained in the presence of various extrinsic cytokines, we labeled each attractor according to the active transcription factors and intrinsic cytokines. Resting CD4+ T cells (labeled Th0) were defined as expressing no transcription factors or regulatory cytokines. Th1 was defined as Tbet and IFN-γ active [8], Th2 as GATA3 and IL-4 active [8] and GATA3+ (a Th2-like cell type) as GATA3+IL4-[38]. Th17 was defined based on RORγt and STAT3 signaling mediated by IL-6 or IL-21, all of which require the presence of TGF-βe [9–10]. iTreg expressed Foxp3 and TGF-β, IL-10 or both, all of which require the presence of IL-2e [16]. Interestingly, the TSRN model also predicts a novel set of steady states that had not been predicted by previous models but that correspond to reported biological cell types (Fig 3B); for example, Tfh cells with Bcl6 and STAT3 signaling mediated by IL-21 [12]; Th9 cells with IL-9, requiring the presence of TGF-β and extrinsic IL-4 [27]; T regulatory cells, as Foxp3-independent CD4+ T cells (TrFoxp3-) with TGF-β, IL-10 or both, but not Foxp3 [52]; Th1 regulatory cells (Th1R) expressing a regulatory cytokine and T-bet [46]; and Th2 regulatory cells (Th2rR) expressing a regulatory cytokine and GATA3 [47]. The model does not consider the Th22 cell type [53] because IL-22 was not included in the network due to the lack of experimental data on this molecule.

To validate the model with experimental data, we simulated loss and gain of function alterations for some nodes. In general, the results agree with the available experimental data, except in the case of the IL-2 knock-out. IL-2- causes the loss of iTreg cells as these cells require continuous IL-2 signaling [54,55], but this differs from the actual IL-2 KO mutants, which lose most CD4+ T cell types because IL-2 is also critical for the activation and survival of CD4+ T cells. This model also allows us to predict the behavior of the Tr Foxp3-, Th1R and Th2R cell types in response to various knock-out and over-expression simulations for several transcription factors or signaling pathways where no experimental data are available.

We performed a functional robustness analysis in which the logical functions of the network were altered (S2 Fig) to verify the construction of the functions and the structural properties of the model and to avoid over-fitting. Altering one of the functions of the network resulted in 1.389% of the initial states attaining a different final attractor than the original final state, and only 0.219% of the initial states arrived at an attractor that was not in the original set of attractors of the non-altered network.

To further verify that the results of the Boolean network are not an artifact due to the discrete nature of the model and to further assess the robustness of the attractors to variations in the node values, we approximated the discrete step-like functions of the Boolean model with continuous interaction functions [44] (S2 File). The continuous model recovers the same attractors as the Boolean regulatory network. Furthermore, these attractors are stable in response to small perturbations in the value of the nodes as predicted by the robustness analyses of the Boolean version of the model.

CD4+ T cell differentiation in response to the micro-environment

Cytokines can be produced by the cell (intrinsic) or by other cells of the immune system (extrinsic). These extrinsic cytokines constitute the micro-environment for CD4+ T cell differentiation. The role of polarizing micro-environments in CD4+ T cell differentiation was assessed using the TSRN model. In this network, the values of the extrinsic cytokines were fixed at a given expression level and the network response was analyzed again (Fig 4). Th0, Th1, Th2 and Tfh can be maintained in the absence of extrinsic cytokines or in the presence of effector cytokines such as IFN-γ, IL-2, IL-4 or IL-21. Th17, iTreg and Th9 cells require extrinsic TGF-β, IL-

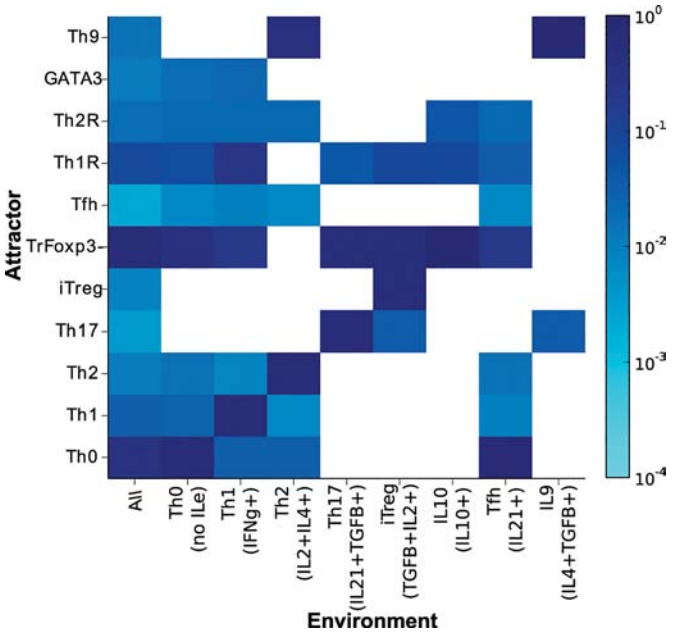


Fig 4. Effect of the micro-environment on CD4+ T cell differentiation as determined using the TSRN model. The values of the extrinsic signals of the TSRN were fixed according to different polarizing micro-environments. The basins of attraction of the resulting attractors were plotted on a logarithmic scale. The following micro-environments were studied: combinations of all extrinsic cytokines, no extrinsic cytokines (Th0), IFN-γe (Th1), IL-4e and IL-2e (Th2), IL-21e and TGF-βe (Th17), TGF-βe and IL-2e (iTreg), IL-10e (IL10), IL-21e (Tfh), and IL-4e and TGF-βe (Th9).

doi:10.1371/journal.pcbi.1004324.g004

2 and IL-4, respectively, to maintain their homeostatic states [13,56]. TrFoxp3- states can be maintained in most polarizing micro-environments [57,58]. The recovered behaviors agree with the experimental data and also with previous models [38].

The importance of the extrinsic cytokines present in the micro-environment can be further analyzed when the system is studied under polarizing conditions. The presence of extrinsic signals for a given cell type increases the number of initial states that differentiate into that cell type, while the absence of extrinsic signals may lead to the loss of a cell type, as is the case with Th17, iTreg and Th9 cells (Fig 4). The presence of the regulatory cytokines IL-10 and TGF-β inhibits most effector CD4+ T cells, except for Th17. This finding may explain the presence of Th17 cells in regulatory micro-environments [59] and provides important insight concerning the relationship between Th17 and iTreg. Thus, this type of modeling framework and analysis may prove useful for finding therapeutic approaches to chronic inflammation.

The polarization of the micro-environment towards a particular cell type increases the size of the basin of attraction and its resistance to transient perturbations. Basin size and attractor stability are not identical (S3 Fig). In this way, the environmental signals promote specific cell types and increase their stability, which likely affects the population dynamics of CD4+ T cells. Nonetheless, different CD4+ T cell types coexist during immune responses. Even if the signals in the micro-environment promote a specific cell type, attractors corresponding to other cell types can still appear in this micro-environment, but their basin sizes and stability tend to be smaller.

CD4+ T cell plasticity in response to the micro-environment

The ability of the immune system to dynamically respond to environmental challenges depends on its plastic responses. CD4+ T cells are phenotypically plastic, and once differentiated,

their expression patterns can be altered depending on internal and external cues. This cell plasticity seems to be important for the overall plasticity of immune system responses [1].

To analyze CD4+ T cell plasticity, we transiently perturbed the attractors of the system. For each attractor we altered the value of one of its nodes and then evaluated the system until an attractor was reached. If the original attractor was reached, we considered the corresponding cell type as stable towards that perturbation. If a new cell-type was reached, we considered that the transition from one cell type to another corresponded to phenotypic plasticity. This analysis was repeated for every node and every attractor. This methodology allowed us determine all the transitions between cell types, the specific perturbation that caused the transition, and the path from one cell-type to another. These transient perturbations in the values of the nodes are equivalent to developmental noise or temporal changes in the micro-environment of the cell. The result is a cell-fate map where the nodes represent CD4+ T cell types recovered by the TSRNand the connections represent the possible transitions between pairs of differentiated cell types (Fig 5, S3 File).

The model recovers the reported transitions corresponding to the polarization of naïve CD4 + T cells into canonical CD4+ T cell types, as well as various events of trans-differentiation between canonical CD4+ T cell types. Most of the predicted transitions are to or from Th0 or towards TrFoxp3-. It is important to clarify that the TCR complex was not included in the minimal model. Thus, in our model, the Th0 attractor represents resting CD4+ T cells. There are few direct transitions among the Th1, Th2, and Th17 cell types. The few direct transitions found towards iTreg and Tfh can only be achieved in polarizing micro-environments. It is also possible to transition from one of the main cell types to another one through the Th0, TrFoxp3-, Th1R, Th2R or GATA3+IL4- attractors. This ability raises multiple questions about the signals necessary for plasticity *in vivo*. It is possible that in order to transition from one cell type to another, some signals have to be maintained for a certain period of time, or that more than one perturbation is necessary. Further studies are required to determine which conditions are necessary and sufficient for CD4+ T cell type transitions to further understand CD4+ T cell plasticity.

Therefore, in the context of this study, we define plasticity as the potential of a given differentiated cell to attain other fates in response to alterations in the expression patterns of their intrinsic components and/or of the extrinsic micro-environment. Of the total of 121 possible transitions between cell types arising from those alterations, the TSRN network yielded 66 cell-type transitions. Thus, the topology or set of regulatory interactions proposed in this network generates restrictions in terms of cell types but also in terms of the patterns of cell-fate transitions.

CD4+ T cells are typically under the influence of particular micro-environments, with specific cytokines affecting the dynamics of these cells. Depending on the combination of cytokines, some cell types are lost, and transitions among the remaining cell types are also restricted. To simulate polarizing micro-environments, we fixed the value of the cytokines associated with pro-Th1 (IFGγe), pro-Th2 pro-(IL-4e, IL-2e), pro-Th17 (IL-21e, TGF-βe), pro-iTreg (TGF-βe, IL-2e), pro-Tr (IL-10,) pro-Tfh (IL-21e) and pro-Th9 (IL-4 and TGF-βe). In general, the polarizing micro-environment increases the size of the attraction basin, the stability and the transition into the attractor. The biological nature of the polarizing signal affects the nature of the resulting transition. In response to regulatory signals (IL-10e, TGF-βe), the majority of the transitions are towards TrFoxp3-, while inflammatory signals lead to more transition signals towards Th1 and Th2. All of these results represent interesting predictions that could be tested experimentally.

Activation of specific CD4+ T transcriptional-signaling regulatory network nodes induces cell type plasticity while inhibitions induce stability. The nature of the perturbation

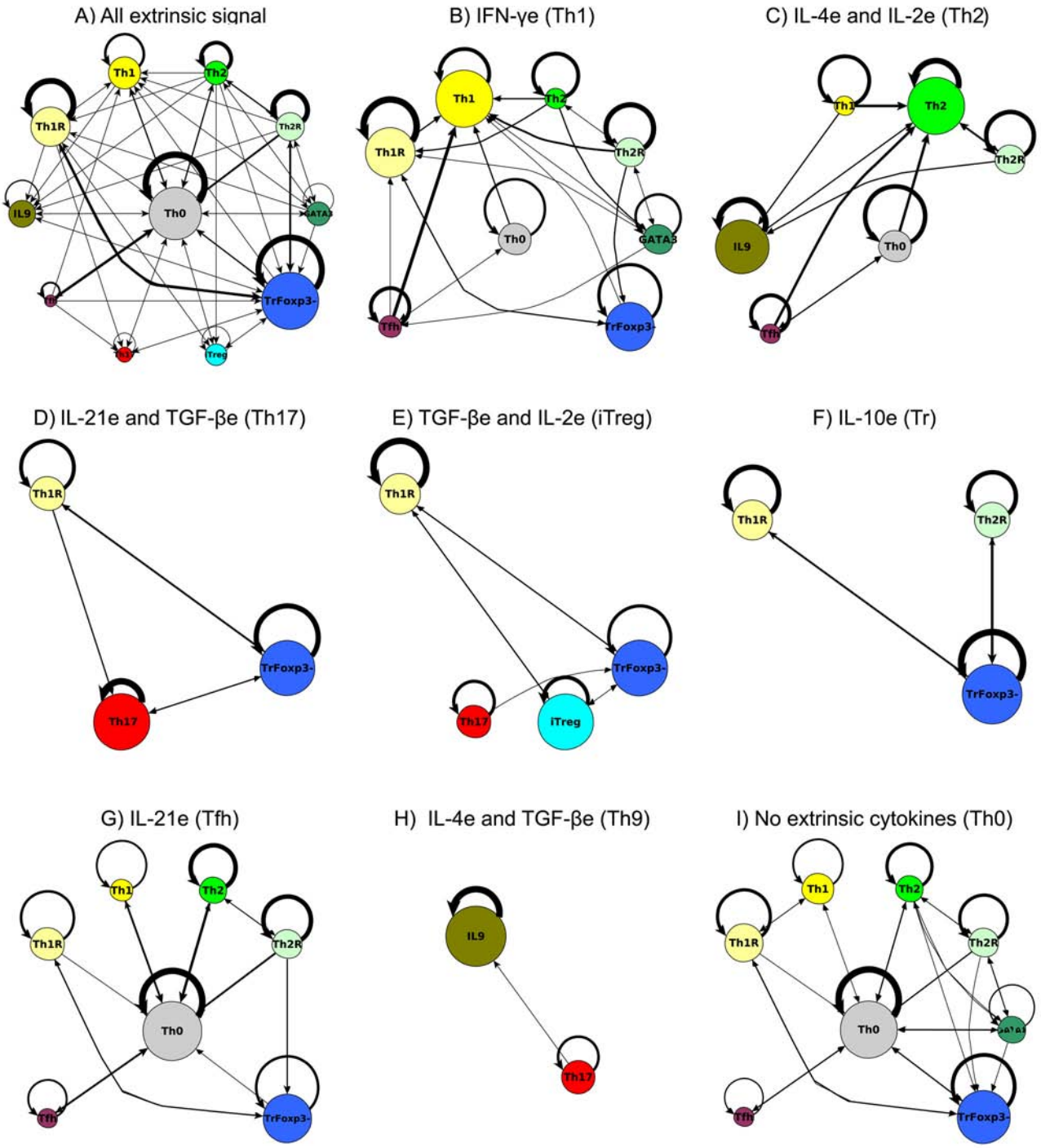


Fig 5. Cell fate map in response to the micro-environment and perturbations of the TSRN model. The values of the extrinsic signals of the TSRN were fixed according to different polarizing micro-environments, and the resulting attractors were transiently perturbed. The nodes represent CD4+ T cell types, and the node sizes correspond to the size of the basin of attraction. The edges represent transitions between cell types, the width of the edges corresponds to the number of times the transition occurred in logarithmic scale, and self-loops correspond to perturbations where the network returned to the original cell type. The following micro-environments were studied: combinations of: **(A)** all extrinsic cytokines, **(B)** IFN-γe (Th1), **(C)**IL-4e and IL-2e (Th2), **(D)** IL-21e and TGF-βe (Th17), **(E)** TGF-βe and IL-2e (iTreg), **(F)** IL-10e (IL10), **(G)** IL-21e (Tfh), **(H)** IL-4e and TGF-βe (Th9), **(I)** no extrinsic cytokines (Th0).

doi:10.1371/journal.pcbi.1004324.g005

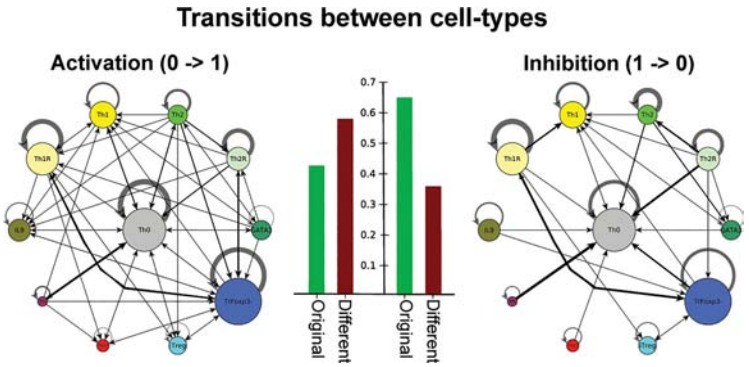


Fig 6. Cell fate map in response to activating or inhibitory signals of the TSRN model. The attractors of the network were transiently perturbed in all possible micro-environments. Perturbations were considered activations (0 → 1) when a previously inactive element was turned on, and inhibitions (1 → 0) when a previously active element was turned off. The nodes represent CD4+ T cell types, and the node sizes correspond to the size of the basin of attraction. The edges represent transitions between cell types, the width of the edges correspond to the number of times the transition occurred on the logarithmic scale. The number of transitions towards a different or the original cell type were counted for both activations and inactivations.

doi:10.1371/journal.pcbi.1004324.g006

is also important for CD4+ T cell plastic responses or stability (Fig 6). If an inactive node is activated (0→1), there is a high probability that a transition from one cell type to another is induced. In contrast, if an active node is inactivated (1→0), there is a high probability that the system remains in the original cell-type. This pattern may be caused by the topology of the network and, in particular, may depend on the functional feedback loops of the system that are altered. The positive feedback loops of a cell type may increase the stability of an attractor and help to recover a transiently inactive node, thus stabilizing a given differentiated state. We hypothesize that the activation of a previously inactive node may induce more transitions, as this alteration likely affects the positive and negative functional circuits of the system [60], thus increasing the chances that the system leads to a new attractor. Further simulations should be used to exhaustively test how specific regulatory circuits react to transient activations and inhibitions. In any case, the analysis presented in this study enables us to postulate that CD4+ T cells are expected to be able to react to activation signals and environmental alterations but are stabilized in response to the transient loss of signals. Thus, the proposed model for CD4+ T cell dynamics implies that these cells are under an unstable equilibrium between cell-fate stability and plasticity.

Key nodes for CD4+ T transcriptional-signaling regulatory network plasticity

While all the elements of the TSRN have previously been shown to be necessary for the differentiation of CD4+ T cells, we wished to address their relative importance in cell plasticity responses. To evaluate this question, we perturbed each node of all the attractors and measured how many times the perturbed state changed to a new attractor (Fig 7) and to which new cell type the system converged (S4 Fig). This process is equivalent to the temporal activation or inactivation of a transcription factor or an element of the signaling pathway in response to noise. Alterations of T-bet and TGF-β usually caused the perturbed state to change from one attractor to another, while RORγt and IL-9 had the least effect on cell-fate transitions. In general, the system is more sensitive to perturbations in the master transcriptional regulators than to alterations of the cytokines.

In contrast to previously published T CD4+ network models that only included SOCS1 [37–40], several SOCS-type proteins were considered in the TSRN presented and analyzed here. SOCS proteins are important for the differentiation and plasticity of CD4+ T cells. SOCS1 is commonly silenced in inflammatory diseases, and over-expression of SOCS3 correlates with allergies [31,51]. To explore the role of SOCS proteins and the impact of alterations in these proteins on CD4+ T type transitions, we generated a network lacking the inhibitions mediated by these proteins (Fig 8). This altered system recovers the original attractors including Th0, Th1, Th2, Th17, iTreg, Tfh, TrFoxp3-, and Th9, but it also predicts novel attractors expressing RORγt+IL-10+ (Th17R) and GATA3+IL-10+IL-9+ (Th2RIL9+), thus confirming the importance of SOCS proteins for attaining the Th17 and Th9 cell types. The importance of IL-10 for CD4+ T cell plasticity dramatically increased in the altered network, while the importance of the rest of the molecular elements decreased. This result suggests that SOCS proteins play an important role in stabilizing effector cell types and regulating the Th0 and TrFoxp3- cell types. SOCS proteins inhibit signal transduction; IL-10 in particular acts through these proteins to regulate CD4+ T cells. This regulation is important to buffer the effect of extrinsic cytokines in the TSRN network model. When SOCS proteins are absent, the network is more sensitive to changes in extrinsic cytokines and IL-10. Further analyses of the effects of SOCS proteins on CD4+ T cells and the possibility of updates to the model based on experimental work should enable the evaluation of more subtle alterations in and combinations of SOCS proteins.

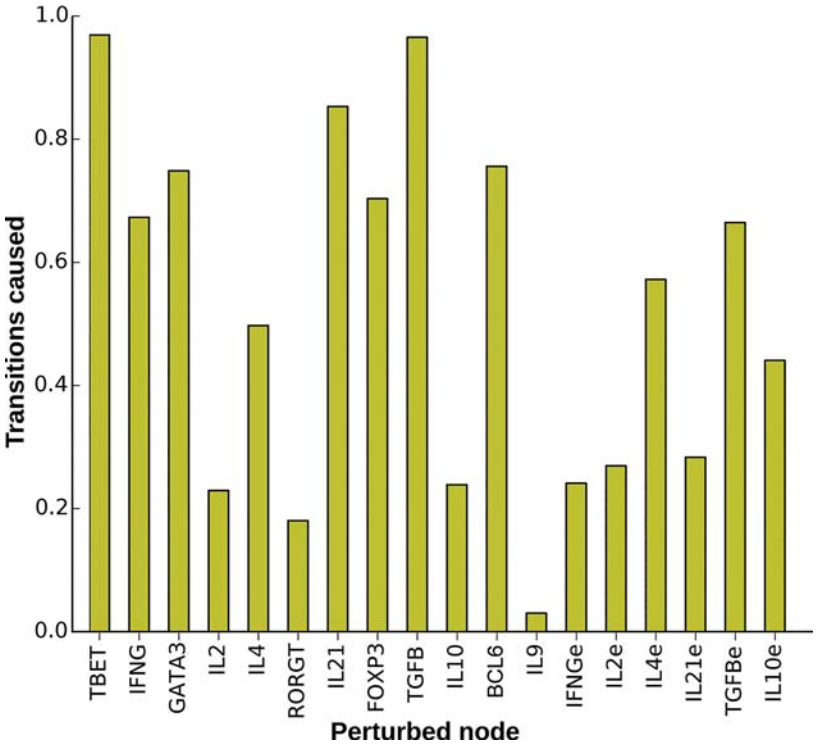


Fig 7. Role of different network nodes in the plasticity of the TSRN model. The proportion of transitions between attractors in response to transient perturbations in the value of each node. On average, 37.76% of the perturbations result in transitions to another cell type, with 47.12% of perturbations of intrinsic components resulting in transitions, compared with 24.43% of perturbations of extrinsic cytokines.

doi:10.1371/journal.pcbi.1004324.g007

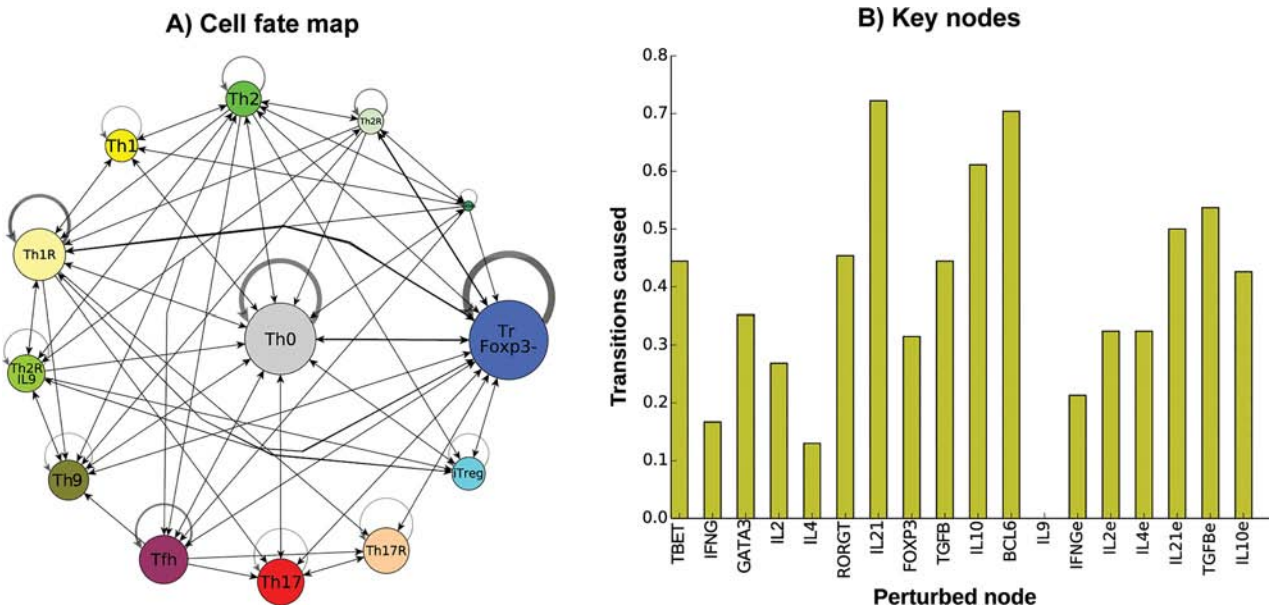


Fig 8. Role of SOCS proteins in the differentiation and plasticity of the TSRN model. The interactions mediated by SOCS proteins were removed to study their role. **(A)** Cell fate map of CD4+ T cell types when the SOCS protein interactions are removed from the TSRN model. The nodes represent CD4+ T cell types and the node sizes correspond to the size of the basin of attraction. New attractors corresponding to GATA3+IL9+IL10+ (Th2RTh9) and RORγt+IL-10+ (Th17R) appeared. The edges represent transitions between cell types, the width of the edges corresponds to the number of times the transition occurred on logarithmic scale, and self-loops correspond to perturbations where the network returned to the original cell type. **(B)** Proportion of transitions between cell types in response to transient perturbations in the value of each node. On average, 21.65% of the perturbations result in transitions to another cell type, with 17.55% of perturbations of the intrinsic components of the network resulting in transitions, compared with 27.51% of perturbations of extrinsic cytokines.

doi:10.1371/journal.pcbi.1004324.g008

Discussion

This model provides a mechanistic description of the way in which CD4+ T cell types and plasticity emerge from the interactions among the intrinsic and extrinsic components of the immune response. The study formally shows that, as expected, the interactions among master transcription factors considered in the TSRN are not sufficient to recover the configurations characteristic of CD4+ T cell types, nor the reported transition patterns. Furthermore, these results clearly demonstrate the necessity to include the feedback from signaling pathways in response to cytokines to recover most of the range of CD4+ T cell types (Th0, Th1, Th2, Th17, Tfh, Th9, iTreg and T regulatory Foxp3 independent cells) and their transition pathways.

As noted above, CD4+ T cell differentiation does not arise solely from the regulatory action of the core of the reported "master" transcription factors (TF): T-bet, GATA3, Foxp3, RORγt and Bcl6. This may be due to the lack of feedforward loops mediated by the transcription factors RORγt [48] and Bcl6 [49,50]. These results show that the transcriptional regulatory core of CD4+ T cell differentiation is necessary, but not sufficient for CD4+ T cell differentiation. The emergence of the different CD4+ T cell types and their transition patterns, requires the feedback from cytokine signaling pathways and external cues.

This model provides a formal test for the emergence of different CD4+ T cell types from feedback or cooperative dynamics among master transcriptional factors, signaling pathway, cytokines produced by the cell and those present in the micro-environment. The proposed model recovers the observed configurations for the following CD4+ T cell types: Th0, Th1, Th2, Th17, Tfh, Th9, iTreg and T regulatory Foxp3 independent cells [2,4–7]. The model also yields

the cell types Tfh, Th9 and T regulatory Foxp3 independent cells that had not been previously incorporated into such models [37–40].

CD4+ T cell types depend on signals from other cells for their differentiation and maintenance. The cytokines in the micro-environment restrict which cell types and transitions can be attained. A cytokine micro-environment that promotes a particular cell type increases its attraction basin size, stability and increases the number of transitions towards the promoted cell type. Nonetheless, different CD4+ T cell types can coexist in micro-environments that do not promote all the present cell types. For example, the presence of pro-regulatory cytokines IL-10 and TGF-β inhibits most effector cells, except for Th17. This finding may explain the presence of Th17 cells in regulatory micro-environments [59] and provides important insights concerning the relationship between Th17 and iTreg cells and the paradoxical role of TGF-β in inflammation [61]. Thus, the type of modeling framework and analyses presented here may prove to be useful for efforts to find therapeutic approaches to address chronic inflammation.

The model was also used to analyze the plasticity of CD4+ T cells by systematically testing how transient perturbations affect the transition patterns among cell types under various micro-environments. Previous studies focused on cell plasticity in response to different micro-environments [38] or on the role of specific molecules [40], rather than studying these phenomena as consequences of the global properties of the system. For example, the TSRN faithfully captures the polarization of resting CD4+ T cells into Th1, Th2, Th17, iTreg and Foxp3-independent T cells, but the predicted cell-fate maps lack direct transitions from iTreg to Th17 and Th17 to Th1 [23–25]. The TSRN model may lack components, interactions or epigenetic mechanisms of regulation that are important to enabling such direct transitions [33]. An additional possibility is that signals must be combined during particular lengths of time to enable some transitions. Further theoretical and experimental research is required to understand the mechanisms underlying CD4+ T cell plasticity. However, the qualitative model proposed here can serve as a framework to incorporate additional details involved in CD4+ T plasticity.

Our model shows that the activation of specific CD4+ T cell transcriptional-signaling regulatory network nodes generally induce cell type plasticity while inhibitions induce stability. The observed response patterns may be caused by the feedback loops and mutual inhibitions molecular network. These findings are coherent with the fact that the immune system generates a specific immunological response to particular challenges, maintains this response while the challenge remains present, and finally downregulates the immune response once the challenge has passed, thus maintaining homeostasis [3,61].

Our model suggests that T-bet, TGF-β and SOCS proteins are key network components to recover the observed CD4+ T cell plasticity. Although T-bet is a key transcription factor for Th1, it also inhibits other transcription factors regulating the differentiation into different cell types [4]. TGF-β is a critical regulator of the immune response but also plays a key role during chronic inflammatory responses [61]. SOCS proteins regulate the phosphorylation of STAT proteins, playing a key role in modulating the signal transduction among different cell types [31,51]. Determining the key elements enabling cell-type plasticity has possible therapeutic implications, as these findings can help to identify therapeutic targets for modulating the immune response while predicting and avoiding secondary effects[3,62].

Given the complexity of CD4+ T cell expression patterns and transitions, it remains unclear whether cytokine expression profiles correspond to lineages or subsets [1–3,22]. The term lineage implies the stability of the cellular phenotype and that the cell has committed to an expression pattern and will maintain it in a fairly robust manner, regardless of environmental alterations. On the other hand, the term subset implies that the cell has a specified expression pattern but that extrinsic signals are required to maintain that pattern [1,22]. Cell types Th1,

Th2, Tfh and TrFoxp3- can be considered lineages, as they exhibit commitment under different cytokine milieus, even if the extrinsic signals change, although environmental alterations can still affect their stability. However, Th17, iTreg and Th9 cells, which require TGF-βe, IL-2e or IL-4e respectively, are potentially subsets. Th17 and iTreg cells also have small basins of attraction, low stability, and require extrinsic signals, exhibiting a lack of commitment. Th9 has a larger basin of attraction than Th17 or iTreg, but is less stable and susceptible to environmental alterations. Based on our analyses, we propose that the degree of dependence on extrinsic signals and the stability in response to changes in the micro-environment can provide clearer and more objective criteria to distinguish between CD4+ T cell subsets and lineages.

CD4+ T cell differentiation and plasticity arises from the feedback among multiple levels of regulation: transcriptional regulation, signaling pathways and the micro-environment. Studying the molecular network as a dynamic system allows us to understand how the interactions among the components, the topology of the network, and the dynamic functions of the nodes give rise to the biological behavior. However, further theoretical and experimental research is required to understand CD4+ T cells. As our understanding of these cells improves, it will be possible to incorporate more detailed molecular information, such as the effect of relative expression levels and the characteristic time courses of expression in the system. This will, in turn, allow us to recover novel cell types and their relationship with other CD4+ T cell types and other cells of the immune system. The present model can now be extended to incorporate multiple cells and their population dynamics [39], relationships with other cells of the immune system, and the formation of specialized niches that result from the dynamic interaction with the micro-environment. This approach will allow us to differentiate between CD4+ T cell subsets and lineages, to understand the developmental dynamics between the different cell types, and to propose approaches to immune system reprogramming that can be used in the clinic.

Methods

Logical modeling formalism: Boolean networks

CD4+ T cell differentiation results from interactions among cytokines, signaling pathways and transcription factors. These interactions were modeled using Boolean networks that enabled us to integrate the qualitative nature of complex regulatory systems. A Boolean network is composed of nodes that represent the system´s molecular components (i.e., cytokines, signaling pathways or transcription factors). In a Boolean network, each node represents a component (gene, protein, phenomenological signal) that can be associated with a discrete variable denoting its current functional level of activity. If the node is functional its value is 1, and if it is not functional, then its value is 0 (see S1 File). Some nodes required special considerations concerning their activation states in the Boolean model. For example, in the case of GATA3, which is continuously expressed during T-cell-lineage development and is necessary for lineage commitment and maintenance, $GATA3^{low}$ is set to 0. As GATA3 is upregulated in Th2 differentiation [63], we set $GATA3^{high}$ to 1. Another example concerns STAT proteins, which are activated when the protein is phosphorylated, forming a dimer that translocates to the nucleus, where it activates its target genes. In this case, the value for STAT protein activation was only set to 1 when all the required conditions were met.

The value of a node xi at a time t depends on the value of the input nodes (including itself), referred to as its regulators. This value can be expressed with a logical function that describes the behavior of the node through time:

$$x_i(t) = \phi_\iota(\tau, \xi_1, \xi_2, \xi_2, \ldots, \xi_\iota, \ldots, \xi_\nu).$$

Weak interactions that are not necessary or sufficient, but only modulate a target factor, were not included in the input regulators of the truth tables (S1 File). Such is the case for Foxp3, which positively modulates the expression of IL-2Rα, which can be activated and functional in the absence of Foxp3 [64].

An input is a node that affects the values of the network but is independent of the network. The state of the network S can be represented by a vector that specifies the value of each node. The state of the network can be represented by a vector S composed of the values of all the nodes of the system. The state of the network corresponds to the expression patterns of a cell.

Inference of the regulatory functions. Boolean functions were defined based on the available experimental data for the reported interactions among a network of 85 components (S1 Table). A transcription factor regulates another factor if it binds to the regulatory region of the latter factor and inhibits or activates is transcription. A cytokine is present if it is either secreted by the cell (intrinsic) or produced by other cells of the immune system (extrinsic). To separate the effects of the cytokines produced by the immune system from those of the cytokines produced by the CD4+ T cell, we label extrinsic cytokines as ILe. Receptors are considered to be active when the cytokine is stably bound to a receptor, enabling it to transduce a signal. STAT proteins are considered active when they are phosphorylated and capable of translocating to the nucleus. The activation of a STAT protein depends on the presence of interleukin, its correct binding to the receptor, and subsequent phosphorylation. SOCS proteins inhibit the phosphorylation of STAT by competing for the phosphorylation site.

Model reduction

To facilitate the analysis of the network and determine which components were necessary and sufficient to recover observed profiles and their patterns of transition, we reduced the extended regulatory network consisting of 85 nodes to one with 18 nodes, including 5 transcription factors, 7 signaling pathways and 6 extrinsic cytokines. To simplify the network, we assumed that the signal produced by the TCR and its co-factors was constitutive and ignored weak interactions as well as input and output nodes. Considering that the expression level of node x_i at time t is represented by $x_i(t)$, the attractors (steady states) that represent different phenotypes are determined by: $x_i(t+1) = x_i(t)$.

In that case, the mapping becomes a set of coupled Boolean algebraic equations. The explicit expressions of the attractors are then obtained by performing the algebraic operations according to the axioms of Boolean algebra [44]. Self-regulated nodes were not removed. If a node was removed, then the logical rules of its targets were modified, maintaining the regulatory logic and indirect regulation. To verify that we did not remove a necessary node, we recovered the attractors of the network and ensured that the configurations corresponding to the Th0, Th1, Th2, Th17 and iTreg states could still be attained (see the details of the reduction methods used in S1 File).

The reduction was verified using the GINsim[45] software. GINsim uses decision diagrams to iteratively remove regulatory components and updates the components to maintain the indirect effects. This method preserves the dynamic properties of the original model. The simplification with GINsim returned a similar network to the one that we obtained with the Boolean logic reduction method proposed by Villareal et al. ([44];S1 File).

Dynamic analysis

After inferring and simplifying the network, we studied its dynamic behavior. A regulatory network is a dynamic system. The state of a network will change over time depending on the

logical functions associated with each node. When the values of a state vector S at t+1 are the same as those at time t, the system has attained an attractor: $S^*(t) = S(t+n)$, $n \geq 1$.

An attractor is interpreted as a stable expression phenotype of a cell, representing a cell type. All the states that lead to a solution S^* constitute the basin of attraction of such an attractor. We determined the attractors and basins of attraction of the network using the R library BoolNet. Attractors were classified depending on the expression of both the master transcription factors and the main cytokine. Th0 was defined as expressing no transcription factors or regulatory cytokines. Th1 was defined as Tbet and IFN-γ active [8], Th2 as GATA3 and IL-4 active [8] and GATA3+ (a Th2-like cell type) as GATA3+IL4-[38]. Th17 was identified by RORγt and STAT3 signaling mediated by IL-6 or IL-21, all of which require the presence of TGF-βe [9–10]. The iTreg type was defined by Foxp3 and TGF-β, IL-10 or both, all of which require the presence of IL-2e [16]. Tfh cells were defined by Bcl6 and STAT3 signaling mediated by IL-21 [12]. Th9 cells express IL-9, requiring the presence of TGF-β and extrinsic IL-4 [27]. T regulatory Foxp3-independent CD4+ T cells (TrFoxp3-) featured TGF-β, IL-10 or both, without expressing Foxp3 [52]. Th1 regulatory cells (Th1R) express a regulatory cytokine and T-bet [46]. Th2 regulatory cells (Th2rR) express a regulatory cytokine and GATA3 [47].

Network validation. The network was validated by comparing it with reported knock-out and over-expression profiles. To simulate loss of function mutations (knock-out) and inhibitions of the signaling pathway, we set the value of the corresponding node to 0 throughout the complete simulation. To simulate over-expression, the value of the node was set to 1.

The functional robustness of the network was characterized by altering the logical functions of the network. Functional robustness refers to the invariance of the attractors in response to noise or perturbations [44]. In this case, to verify that the results of the model did not depend on over-fitting the logical functions, we perturbed the latter and verified the stability of the resulting attractors and their basins. To achieve this, we randomly selected a large number of entries and flipped their values from 0 to 1 or vice versa, one by one (bit flip). The basins and attractors were obtained for the altered networks and compared the original basins and attractors.

To further evaluate the robustness of the network to small changes in the values of the nodes and interaction functions, we approximated the Boolean step functions as continuous functions [44]. We replaced the logical functions $f(x_i)$ with a set of continuous functions that satisfy Zadeh's rules of fuzzy propositional calculus. Using this approach for each state variable, we derived a continuous function, $w_i(q)$. The latter functions correspond to step-like (differentiable) activation functions. The continuous system can then be described by:

$$\frac{dq_i}{dt} = \frac{1}{e^{[-2b(w_i(q)-w_i^{thr})]+1}} 18$$

where w_i is the input function for node i, w_i^{thr} is a threshold level, b is the input saturation rate, and α_i is its relaxation rate. In particular, for $b >> 1$, the activation function becomes a Heaviside step function.

Plasticity

The attractors of the network correspond to cell types. A multi-stable system can have multiple attractors and switch between them in response to alterations in the state of the system [65]. To study the plasticity and robustness of the system we transiently perturbed the attractors of the network and then evaluated the functions until we arrived at an attractor. This methodology enabled us to obtain all the transitions between cell types, the specific perturbations that caused those transitions, and the path from one cell-type to another. We define an attractor as

stable when the system remains in the same attractor in the presence of perturbations. The stability of each attractor in response to changes in the micro-environment and signaling pathways was analyzed by characterizing the evolution of the network in response to pulses of activation or inhibition of specific nodes. To quantify the stability of the attractors of the network, we perturbed the state vector of the solutions for one time step. Then, we counted how many of the perturbed state vectors stayed in the same attractor to quantify its stability. A system is plastic when it can transition from one state to another in response to alterations of the system. More specifically, the network was said to be plastic when a transition occurred from a given attractor to another in response to a transient perturbation in the value of one of its nodes.

Supporting Information

S1 Table. T CD4+ lymphocyte extended regulatory network references.
(XLS)

S2 Table. T CD4+ lymphocyte transcriptional regulatory network model.
(XLS)

S3 Table. T CD4+ lymphocyte transcriptional-signaling regulatory network model.
(XLS)

S1 File. T CD4+ lymphocyte extended regulatory network simplification.
(PDF)

S2 File. T CD4+ lymphocyte transcriptional-signaling continuous regulatory network model.
(PDF)

S3 File. Transitions in response to transient perturbations in the nodes of the T CD4+ lymphocyte transcriptional-signaling regulatory network.
(PDF)

S1 Fig. T CD4+ lymphocyte extended regulatory network.
(EPS)

S2 Fig. Validation of the T CD4+ lymphocyte transcriptional-signaling regulatory network. (A) To validate the TSRN model, we simulated loss of function or null mutations (KO) and over-expression experiments and compared the results with the available experimental data. The values of the nodes were set to "0" for simulations of loss-of-function or knock-out experiments and to "1" for over-expression. The color corresponds to the basin size of each attractor on the logarithmic scale. '—-' represents attractors that were not attained in the original wild type (WT) network. The attractors marked with (red) "X" correspond to incorrect predictions. (B) To verify the construction of the functions and the structural properties of the model, we performed a robustness analysis altering the update rules. Networks with perturbed functions of the TSRN were generated to test the robustness of the structural properties of the networks to noise, mis-measurements and incorrect interpretations of the data. After altering one of the functions of the network, 1.389% of the possible initial states changed their final attractor (yellow), and only 0.219% of the possible initial states arrived at an attractor not present in the original network (red).
(EPS)

S3 Fig. Effect of the environment on the stability of the T CD4+ lymphocyte transcriptional-signaling regulatory network. The values of the extrinsic signals of the TSRN were fixed

according to different polarizing micro-environments. Each attractor was transiently perturbed, and the proportion of transitions that stayed in the same cell type was plotted on a logarithmic scale. The following micro-environments were studied here: combinations of all extrinsic cytokines, no extrinsic cytokines (Th0), IFN-γe (Th1), IL-4e and IL-2e (Th2), IL-21e and TGF-βe (Th17), TGF-βe and IL-2e (iTreg), IL-10e (IL10), IL-21e (Tfh), and IL-4e and TGF-βe (Th9).
(EPS)

S4 Fig. Effect of transient perturbations on the state of the nodes of the T CD4+ lymphocyte transcriptional-signaling regulatory network. Number of transitions to an attractor in response to transient perturbations in the value of each node. The states of the node were perturbed during one time step from 1 to 0 (-) or 0 to 1 (+), depending on its state in the original attractor.
(EPS)

Acknowledgments

This work is presented in partial fulfillment towards Mariana Martínez-Sanchez' doctoral degree in the program "Doctorado en Ciencias Biomédicas, de la Universidad Nacional Autónoma de México". We acknowledge Diana Romo for her help with many logistical tasks.

Author Contributions

Conceived and designed the experiments: ERAB MEMS. Performed the experiments: MEMS CV. Analyzed the data: ERAB MEMS LM CV. Contributed reagents/materials/analysis tools: MEMS. Wrote the paper: MEMS ERAB.

References

1. Bluestone JA, Mackay CR, O'Shea JJ, Stockinger B. The functional plasticity of T cell subsets. Nature Reviews. 2009; 9(11):811–816. doi: 10.1038/nri2654 PMID: 19809471
2. Sallusto F, Monticelli S. The many faces of CD4 T cells: roles in immunity and disease. Seminars in immunology. 2013 Nov; 25(4):249–51. doi: 10.1016/j.smim.2013.11.001 PMID: 24262948
3. Zak DE, Tam VC, Aderem A. Systems-level analysis of innate immunity. Annual review of immunology. 2014 Jan; 32:547–77. doi: 10.1146/annurev-immunol-032713-120254 PMID: 24655298
4. Zhu J, Yamane H, Paul WEW, WE ZJYHP. Differentiation of effector CD4 T cell populations. Annual review of immunology. 2010 Jan; 28(1):445–89.
5. Jiang S, Dong C. A complex issue on CD4(+) T-cell subsets. Immunological reviews. 2013 Mar; 252 (1):5–11. doi: 10.1111/imr.12041 PMID: 23405891
6. Vahedi G, Kanno Y, Sartorelli V, O'Shea JJ. Transcription factors and CD4 T cells seeking identity: masters, minions, setters and spikers. Immunology. 2013 Jul; 139(3):294–8. doi: 10.1111/imm.12113 PMID: 23586907
7. Yamane H, Paul WE. Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. Immunological reviews. 2013 Mar; 252(1):12–23. doi: 10.1111/imr.12032 PMID: 23405892
8. Mosmann TR Cherwinski H. Two types of murine helper T cell clone. I Definition according to profiles of lymphokine activities and secreted proteins J Immunol. 1986; 136:2348–2357. PMID: 2419430
9. Onishi RM, Gaffen SL. Mechanisms of Interleukin-17 and its target interleukin-17 function in disease. Immunology. 2010 Mar; 129(3):311–321. doi: 10.1111/j.1365-2567.2009.03240.x PMID: 20409152
10. Kolls JK, Khader SA. The role of Th17 cytokines in primary mucosal immunity. Cytokine & growth factor reviews. 2010 Dec; 21(6):443–448.
11. Breitfeld D, Ohl L, Kremmer E, Ellwart J, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. The Journal of experimental medicine. 2000 Dec; 192(11):1545–52. PMID: 11104797

12. Choi YS, Yang Ja, Crotty S. Dynamic regulation of Bcl6 in follicular helper CD4 T (Tfh) cells. Current opinion in immunology. 2013 Jun; 25(3):366–72. doi: 10.1016/j.coi.2013.04.003 PMID: 23688737

13. Putheti P, Awasthi A, Popoola J, Gao W, Strom TB. Human CD4 memory T cells can become CD4+IL-9+ T cells. PloS one. 2010 Jan; 5(1):e8706. doi: 10.1371/journal.pone.0008706 PMID: 20090929

14. Lu Y, Hong S, Li H, Park J, Hong B, Wang L, et al. Th9 cells promote antitumor immune responses in vivo. The Journal of clinical investigation. 2012 Nov; 122(11):4160–71. doi: 10.1172/JCI65459 PMID: 23064366

15. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic Self-Tolerance Maintained by Activated T cells Expressing IL-2 Receptor alpha-Chains (CD25). The Journal of Immunology. 1995 Aug; 155(3):1151. PMID: 7636184

16. Rudensky AY. Regulatory T cells and Foxp3. Immunological reviews. 2011 May; 241(1):260–268. doi: 10.1111/j.1600-065X.2011.01018.x PMID: 21488902

17. Rutz S, Ouyang W. Regulation of interleukin-10 and interleukin-22 expression in T helper cells. Current opinion in immunology. 2011 Oct; 23(5):605–12. doi: 10.1016/j.coi.2011.07.018 PMID: 21862302

18. Goswami R, Kaplan MH. A brief history of IL-9. Journal of immunology (Baltimore, Md: 1950). 2011 Mar; 186(6):3283–8. doi: 10.4049/jimmunol.1003049 PMID: 21368237

19. Li H, Rostami A. IL-9: basic biology, signaling pathways in CD4+ T cells and implications for autoimmunity. Journal of neuroimmune pharmacology: the official journal of the Society on NeuroImmune Pharmacology. 2010 Jun; 5(2):198–209. doi: 10.1007/s11481-009-9186-y PMID: 20020328

20. Shin K, Wang YH, Santori FR, Boggiano C, Arima K, Bover L, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. Proceedings of the National Academy of Sciences of the United States of America. 2009 Mar; 106(12):4793–8. doi: 10.1073/pnas.0900408106 PMID: 19273860

21. Murphy KM, Stockinger B, Manuscript A. Effector T cell plasticity: flexibility in the face of changing circumstances. Nature immunology. 2010 Aug; 11(8):674–680. doi: 10.1038/ni.1899 PMID: 20644573

22. O'Shea J, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science (New York, NY). 2010 Feb; 327(5969):1098–102. doi: 10.1126/science.1178334 PMID: 20185720

23. Nakayamada S, Takahashi H, Kanno Y, O'Shea JJ. Helper T cell diversity and plasticity. Current Opinion in Immunology. 2012 Jun; 24(3):297–302. doi: 10.1016/j.coi.2012.01.014 PMID: 22341735

24. Muranski P, Restifo NP. Essentials of Th17 cell commitment and plasticity. Blood. 2013 Mar; 121 (13):2402–14. doi: 10.1182/blood-2012-09-378653 PMID: 23325835

25. Hirota K, Duarte JaH, Veldhoen M, Hornsby E, Li Y, Cua DJ, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. Nature immunology. 2011 Mar; 12(3):255–63. doi: 10.1038/ni.1993 PMID: 21278737

26. Kleinewietfeld M, Hafler DA. The plasticity of human Treg and Th17 cells and its role in autoimmunity. Seminars in immunology. 2013 Nov; 25(4):305–12. doi: 10.1016/j.smim.2013.10.009 PMID: 24211039

27. Kaplan MH. Th9 cells: differentiation and disease. Immunological reviews. 2013 Mar; 252(1):104–15. doi: 10.1111/imr.12028 PMID: 23405898

28. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity. 2008 Jul; 29(1):138–149. doi: 10.1016/j.immuni.2008.05.009 PMID: 18599325

29. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 2008 Jul; 29(1):44–56. doi: 10.1016/j.immuni.2008.05.007 PMID: 18585065

30. Zaretsky AG, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ, et al. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. The Journal of experimental medicine. 2009 May; 206(5):991–999. doi: 10.1084/jem.20090303 PMID: 19380637

31. Yoshimura A, Suzuki M, Sakaguchi R, Hanada T, Yasukawa H. SOCS, Inflammation, and Autoimmunity. Frontiers in Immunology. 2012; 3(March):1–9. doi: 10.3389/fimmu.2012.00001 PMID: 22679445

32. Cobbold SP, Adams E, Nolan KF, Regateiro FS, Waldmann H. Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link. Immunological reviews. 2010 Jul; 236:203–18. doi: 10.1111/j.1600-065X.2010.00913.x PMID: 20636819

33. Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ, Shea JJO. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. Annual review of immunology. 2012 Jan; 30:707–731. doi: 10.1146/annurev-immunol-020711-075058 PMID: 22224760

34. Albert R, Thakar J. Boolean modeling: a logic-based dynamic approach for understanding signaling and regulatory networks and for making useful predictions. Wiley Interdisciplinary Reviews: Systems Biology and Medicine. 2014 Sep; 6(5):353–369. PMID: 25269159

35. Wang RS, Saadatpour A, Albert R. Boolean modeling in systems biology: an overview of methodology and applications. Physical biology. 2012 Oct; 9(5):055001. doi: 10.1088/1478-3975/9/5/055001 PMID: 23011283

36. Azpeitia E, Benitez M, Padilla-Longoria P, Espinosa-Soto C, AlvarezBuylla ER. Dynamic network-based epistasis analysis: boolean examples. Frontiers in plant science. 2011 Jan; 2:92. doi: 10.3389/fpls.2011.00092 PMID: 22645556

37. Mendoza L, Pardo F. A robust model to describe the differentiation of T-helper cells. Theory in biosciences Theorie in den Biowissenschaften. 2010 Oct;p. 283–293.

38. Naldi A, Carneiro J, Chaouiya C, Thieffry D. Diversity and Plasticity of Th Cell Types Predicted from Regulatory Network Modelling. PLoS Computational Biology. 2010 Sep; 6(9):e1000912. doi: 10.1371/journal.pcbi.1000912 PMID: 20824124

39. Mendoza L. A Virtual Culture of CD4+ T Lymphocytes. Bulletin of mathematical biology. 2013 Jan

40. Carbo A, Hontecillas R, Kronsteiner B, Viladomiu M, Pedragosa M, Lu P, et al. Systems modeling of molecular mechanisms controlling cytokine-driven CD4+ T cell differentiation and phenotype plasticity. PLoS computational biology. 2013 Apr; 9(4):e1003027. doi: 10.1371/journal.pcbi.1003027 PMID: 23592971

41. Chaves M, Albert R. Studying the effect of cell division on expression patterns of the segment polarity genes. Journal of the Royal Society, Interface / the Royal Society. 2008 Aug; 5 Suppl 1:S71–84. doi: 10.1098/rsif.2007.1345.focus PMID: 18434279

42. Mendoza L, Sa Ye, Sanchez-Corrales YE, Alvarez-Buylla ER. The Arabidopsis thaliana flower organ specification gene regulatory network determines a robust differentiation process. Journal of theoretical biology. 2010 Jun; 264(3):971–83. doi: 10.1016/j.jtbi.2010.03.006 PMID: 20303988

43. Felix MA. Caenorhabditis elegans vulval cell fate patterning. Physical biology. 2012 Aug; 9(4):045001. doi: 10.1088/1478-3975/9/4/045001 PMID: 22871570

44. Villarreal C, Padilla-Longoria P, Alvarez-Buylla ER. General Theory of Genotype to Phenotype Mapping: Derivation of Epigenetic Landscapes from N-Node Complex Gene Regulatory Networks. Physical Review Letters. 2012 Sep; 118102(September):1–5.

45. Naldi A, Berenguier D, Fauré A, Lopez F, Thieffry D, Chaouiya C. Logical modelling of regulatory networks with GINsim 2.3. Bio Systems. 2009 Aug; 97(2):134–9. doi: 10.1016/j.biosystems.2009.04.008 PMID: 19426782

46. Koch Ma, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ, et al. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nature immunology. 2009 Jun; 10(6):595–602. doi: 10.1038/ni.1731 PMID: 19412181

47. Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, et al. GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. The Journal of clinical investigation. 2011 Nov; 121(11):4503–4515. doi: 10.1172/JCI57456 PMID: 21965331

48. Ciofani M, Madar A, Galan C, Sellars M, Mace K, Pauli F, et al. A Validated Regulatory Network for Th17 Cell Specification. Cell. 2012 Sep; 151(2):1–15.

49. Zhang N, Bevan MJ. Transforming Growth Factor-β Signaling Controls the Formation and Maintenance of Gut-Resident Memory T Cells by Regulating Migration and Retention. Immunity. 2013 Oct; 39 (4):687–696. doi: 10.1016/j.immuni.2013.08.019 PMID: 24076049

50. Pasqualucci L, Migliazza A, Basso K, Houldsworth J, Chaganti RSK, Dalla-Favera R. Mutations of the BCL6 protooncogene disrupt its negative autoregulation in diffuse large Bcell lymphoma. Blood. 2003 Apr; 101(8):2914–23. PMID: 12515714

51. Ca Knosp, Ja Johnston. Regulation of CD4+ T-cell polarization by suppressor of cytokine signalling proteins. Immunology. 2012 Feb; 135(2):101–11. doi: 10.1111/j.1365-2567.2011.03520.x PMID: 22044277

52. Weiner HL. The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. Nature immunology. 2001 Aug; 2(8):671–2. PMID: 11477400

53. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nature immunology. 2009 Aug; 10 (8):857–863. doi: 10.1038/ni.1767 PMID: 19578369

54. Zhu J Cote-Sierra J GLPWE, Zhu J, Cote-Sierra J, Guo L, Paul WE, WE ZJCSJGLP. Stat5 activation plays a critical role in Th2 8differentiation. Immunity. 2003 Nov; 19(5):739–748. PMID: 14614860

55. et al Kagami S Nakajima H SAHKSK, et al Kagami S Nakajima H SAHKSK. Stat5a regulates T helper cell differentiation by several distinct mechanisms. Blood. 2001; 97:2358–2365. PMID: 11290598

56. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nature immunology. 2008 Jun; 9(6):641–649. doi: 10.1038/ni.1610 PMID: 18454151

57. Carrier Y, Yuan J, Kuchroo VKVKK, Weiner HLHLHL. Th3 Cells in Peripheral Tolerance. I. Induction of Foxp3-Positive Regulatory T Cells by Th3 Cells Derived from TGF-β T Cell-Transgenic Mice. The Journal of Immunology. 2011; 178(1):179.

58. Barnes MJ, Powrie F. Regulatory T cells reinforce intestinal homeostasis. Immunity. 2009 Sep; 31 (3):401–11. doi: 10.1016/j.immuni.2009.08.011 PMID: 19766083

59. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. Cell. 2010 Mar; 140(6):845–858. doi: 10.1016/j.cell.2010.02.021 PMID: 20303875

60. Mendoza L, Thieffry D, Alvarez-Buylla ER. Genetic control of flower morphogenesis in Arabidopsis thaliana: a logical analysis. Bioinformatics. 1999 Jul; 15(7):593–606. PMID: 10341422

61. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, et al. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. Nature immunology. 2007 Dec; 8(12):1390–7. PMID: 17994024

62. Germain RN, Meier-Schellersheim M, Nita-Lazar A, Fraser IDC. Systems biology in immunology: a computational modeling perspective. Annual review of immunology. 2011 Apr; 29:527–85. doi: 10.1146/annurev-immunol-030409-101317 PMID: 21219182

63. Ho IC, Tai TS, Pai SY. GATA3 and the T cell lineage: essential functions before and after T-helper-2-cell differentiation. Nat Rev Immunol. 2009 Feb; 9(2):125–135. doi: 10.1038/nri2476 PMID: 19151747

64. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nature immunology. 2011 Jun; 12(6):551–559. doi: 10.1038/ni.2030 PMID: 21516110

65. van den Ham HJ, de Boer RJ. Cell division curtails helper phenotype plasticity and expedites helper T-cell differentiation. Immunology and cell biology. 2012 Oct; 90(9):860–8. doi: 10.1038/icb.2012.23 PMID: 22565392

Capítulo 3

The CD4+ T cell regulatory network mediates inflammatory responses during acute hyperinsulinemia: a simulation study

The CD4+ T cell regulatory network mediates inflammatory responses during acute hyperinsulinemia: a simulation study

M.E. Martinez-Sanchez[1,2], M. Hiriart[2,3], E. R. Alvarez-Buylla[1,2,*]

[1] Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México.

[2] Centro de Ciencias de la Complejidad, Universidad Nacional Autónoma de México.

[3] Departamento de Neurodesarrollo y Fisiología, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México.

* To whom correspondence should be addressed: eabuylla@gmail.com

Abstract

Obesity is linked to insulin resistance, high insulin levels, chronic inflammation, and alterations in the behavior of CD4+ T cells. Despite the biomedical importance of this condition, the system-level mechanisms that alter CD4+ T cell differentiation and plasticity are not well understood. We model how hyperinsulinemia alters the dynamics of the CD4+ T regulatory network, and this, in turn, modulates cell differentiation and plasticity. Different polarizing micro-environments are simulated under basal and high levels of insulin to assess impacts on cell-fate attainment and robustness in response to transient perturbations. In the presence of high levels of insulin Th1 and Th17 become more stable to transient perturbations and their basin sizes are augmented, IL10 producing regulatory T cells become less stable or disappear, while TGFB producing cells remain unaltered. Hence, the model provides a dynamic system-level explanation for the documented apparently paradoxical role of TGFB in both inflammation and regulation of immune responses and the emergence of the adipose Treg phenotype. Furthermore, our simulations provide novel predictions on the impact of the micro-environment in the coexistence of the different cell types, proposing that in pro-Th1, pro-Th2 and pro-Th17 environments effector and regulatory cells can coexist, but that high levels of insulin severely affect regulatory cells, specially in a pro-Th17 environment. This work provides a system-level formal and dynamic framework to integrate further experimental data in the study of complex inflammatory diseases.

Introduction

Obesity-associated chronic inflammation is a complex phenomenon that results from the interaction between adipose tissue, hyperinsulinemia, and chronic inflammation (1-4). Together, these linked conditions increase the risk to develop metabolic syndrome and type 2 diabetes mellitus. To understand how such complex syndrome emerges, it is necessary to use an integrative, system-level and dynamic approach that takes into consideration: the non-linearity of the interactions, the strong effect of the environment, the constant crosstalk and feed forward interactions among the genetic and non-genetic components involved, and the synchronic or concerted nature of various regulatory events and conditions involved occur (5-8). Most studies have focused on the direct relationship between macrophages and obesity (9), meanwhile, important questions concerning the relationship between obesity, insulin, and CD4+ T cell types populations and plastic changes among them remain unaddressed. These probably play important roles in the onset of inflammatory responses, and their systemic impact remains unresolved. A starting point, involves understanding: (i) the complex regulatory network involved in the cell fate attainment of CD4+ T cell types (10-12), (ii) how such

network responds to extracellular metabolic and environmental conditions (13-14) (iii) how the resulting system modulates the inflammatory and immune responses (1-4).

Obesity-associated chronic inflammation result from prolonged excessive nutrient intake (15, 16). Under such condition, adipocytes in the visceral adipose tissue (VAT) stimulate the inflammatory response by producing pro-inflammatory cytokines, increasing activated macrophages, but also altering the CD4+ T cell population which likely feedbacks to inflammation (15, 17). This inflammatory response causes a decrease in glucose intake, which affects glucose metabolism and may indirectly promote an increase in insulin production by beta cells (1-4, 15, 16, 18). Hyperinsulinemia is strongly associated with metabolic syndrome that is typified by obesity, hypertension, dyslipidemia, renal failure, fatty liver disease, certain cancers and cardiovascular diseases, among others. Despite the fact that such syndrome is clearly characterized, we still do not understand the system-level underlying mechanisms, as well as the global health consequences associated to hyperinsulinemia (18).

CD4+ T cells are fundamental modulators of immune challenges and the homeostasis of the immune system. Naive CD4+ T cells (Th0) are activated when they recognize an antigen in a secondary lymphoid organ. CD4+ T cells may attain different cell fates depending on the cytokine milieu and other signals in their micro-environment. The cytokines can be produced by the lymphocyte (intrinsic) or by other immune cells (extrinsic). The different cell types express characteristic transcription factors and cytokines and have been associated with specific roles in the immune system (19). The classification of CD4+ T cells in subsets has been complicated, as they are highly heterogeneous and plastic. There are reports of hybrid cells that express transcription factors and cytokines from more than one cell type (20, 21), for example. Furthermore, CD4+ T cells can plastically alter their expression patterns in response to environmental conditions (22-24). Such complex and dynamic plastic behavior has started to be explained at the system level using multistable network models (10-12).

Regulatory T cells maintain immune tolerance; regulate lymphocyte homeostasis, activation, and function. Regulatory T cells can be classified into various types. Treg cells are characterized by the transcriptional factor Foxp3, high expression of CD25+, and they produce TGFβ and IL10. But, these two cytokines can also be expressed independently of Foxp3. TGFβ is necessary for the differentiation of regulatory Tregs and effector Th17 cells. TGFβ has a context-specific role in the immune response; it can suppress or enhance the immune reaction, depending on its cofactors (25-27). IL-10 is an immunosuppressive cytokine produced by many cells of the immune response. It acts as a feedback regulator of the immune response by inhibiting the production of inflammatory cytokines (28). Moreover, T cells that express Tbet or GATA-3, in addition to certain regulatory factors, are important in regulating the Th1 and Th2 response (29-31).

CD4+ T cells are involved in the inflammatory feedback loop in obesity-associated tissue inflammation. In the obese VAT murine models and humans, an enrichment of the Th1 and Th17 populations and a decrease in regulatory T cells has been described (3, 32-34). Th1 and Th17 cells produce proinflammatory cytokines that inhibit insulin signaling. The transcriptional profiles and functions of Tregs are also altered, they express proinflammatory cytokines like IFNγ, and IL-10. This change in expression patterns causes Tregs to cluster with inflammatory T cells (3, 32-34). While TGFβ is detectable in adipose tissue, its role in regulating Treg cells is unclear (34). Paradoxically, it has been reported that adipose tissue Treg cells decrease seems to both, improve and worsen insulin resistance (33, 35, 36). Such behavior could be linked to a multistable dynamic underlying system such as that recently proposed to study CD4+ T cell differentiation and plasticity (10-12). On the other hand, the general metabolic state of an individual also affects CD4+ T cells. Obesity is associated with increased insulin levels, which affects CD4+ T cells. Insulin is necessary for the survival and proliferation of activated CD4+ T cells. Effector T cells, such as Th1, Th2 and Th17, depend on glycolysis, while

resting (not activated) regulatory and memory T cells depend mainly on lipid oxidation. But in obese VAT, the high levels of insulin over-activate the AKT pathway, inhibiting IL-10 production and its regulatory functions in CD4+ T cells (14). Hence, the relationship between insulin resistance and CD4+ T cells is still unclear.

We propose here a theoretical simulation study, to explore the molecular interactions between the previously published CD4+ T cell regulatory network (12) and insulinemia (14). Such a system-level mechanistic approach is fundamental for understanding CD4+ T cell differentiation and plasticity dynamics at the cellular level in response to the metabolic state of hyperinsulinemia. We also analyze such altered dynamics of CD4+ T cell dynamics under different IL10 environments. We used the Boolean regulatory network for studying CD4+ T cell differentiation and plasticity dynamics in response to insulin. The system includes transcription factors, signaling pathways, intrinsic and extrinsic cytokines (12), as well as the impact of basal and high levels of insulin (14). The model recovers the differentiation of T CD4+ cells, including effector (Th1, Th2, Th17) and regulatory (iTreg, Th1R, Th2R, Foxp3-IL10+ and Foxp3-TGFβ+ cells) cell types (12,19). Here, we show how hyperinsulinemia shapes CD4+ T cell attainment by reducing the production of IL-10 and causing a shift towards pro-inflammatory, resting, or TGFβ+ producing cell types. Constant pro-regulatory signals can counteract this change. We also explore how the presence of high levels of insulin in the environment alters the plasticity of CD4+ T cell in response to transient fluctuations in the elements of the network. High insulin also favors transitions towards inflammatory, resting or TGFβ+ producing cell types and reduces the stability of regulatory cell types. In this way, we show how the CD4+ T cell molecular network model proposed before (12) seems to mediate the observed cellular behavior in obesity-associated chronic inflammation. This network model constitutes a useful framework to further explore the system-level mechanisms involved in inflammatory conditions including obesity.

Results

CD4+ T cell regulatory network

We expanded the previously published T CD4+ cell transcriptional-signaling regulatory network (12) to include the effect of insulin in the differentiation of CD4+ T cells, according to experimental data (14). The CD4+ T cell differentiation/plasticity network focuses in activated CD4+ T cells in VAT, and was grounded on experimental data [File S1]. Using this model we studied the role of the different network components in the cellular dynamics and the impact of the environment in cell fate attainment and plasticity patterns [Figure 1]. The model focuses on inactivated CD4+ T cells; it assumes that the T cell receptor (TCR) and its cofactors are active, and ignores the differences in glycolysis and lipid oxidation metabolism between effector and regulatory T cells. Furthermore, as the model is a minimal network, various components of the system were simplified, but previous simulations guarantee that the main dynamic regulatory motifs and feedback are considered (12). Given the available data, the model focuses on the observed behaviors in the VAT ignoring the contributions of other tissues. It also focuses on the first stage of hyperinsulinemia, ignoring long term effects, such as those presented under insulin resistance (9, 34, 36). The model does not include the dynamic interaction with adipocytes or macrophages, nor the effect of sexual hormones (15-17).

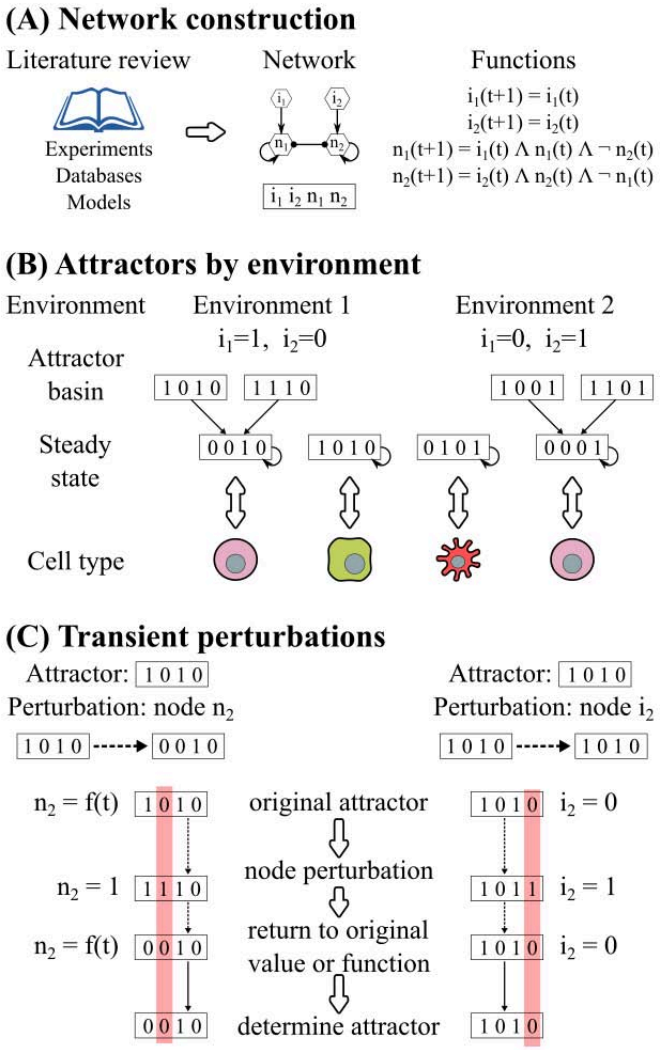


Fig 1. Experimental design of simulations. (A) The network and regulatory functions were grounded on published experimental results. (B) The different inflammatory conditions were simulated by fixing the values of the input nodes of the network, that represent the extrinsic cytokines present in the micro-environment. For each simulated condition, the attractors and basins of attraction of the network were obtained. (C) The attractors of the network were perturbed by fixing the value of the target node for one time step and then returning the node to its original function or value; the system attractor was determined.

The nodes of the network correspond to transcription factors, signaling pathways and cytokines, while the edges correspond to the regulatory interactions between the nodes and are modeled as Boolean functions [Figure 1A; [Table S1]]. The resulting network contains 19 nodes and 54 interactions [Figure 2, BioModelsDatabase: MODEL1606020000]. The nodes include: transcription factors (Tbet, GATA3, RORγt, Foxp3), the effector and regulatory cytokines produced by the cell and their signaling pathways (intrinsic) (IFNγ, IL-2, IL-4, IL-21, TGFβ and IL-10), and the cytokines produced by the rest of the immune system (extrinsic) (IFNγe, IL-2e, IL-4e, IL-10e, IL-12e, IL-21e, IL-27e, and TGFβe). To simulate the effect of hyperinsulinemia we extended the previous network to add the regulation of IL-10 by insulin via the AKT pathway (14); and the STAT3-signaling cytokines: IL-10, IL-6, and IL-21 all use STAT3. We assumed that a different pathway mediates IL-10 signaling than IL-6/IL-21. As the model focuses on activated CD4+ T cells, we assume that the TCR signaling

pathway is constitutively active and did not include explicitly this component in the network. The state of a node represents whether the biological component is active (1) or inactive (0). A node is active when it is capable of altering the regulation of other components of the immune system. For example, as CD4+ T cells require a basal level of insulin to survive, we considered this basal level to have a value of 0, while a higher insulin concentration, that is capable of affecting IL-10, is fixed to 1(14, 37). In other words, hyperinsulinemia is simulated by setting the "insulin" node to 1.

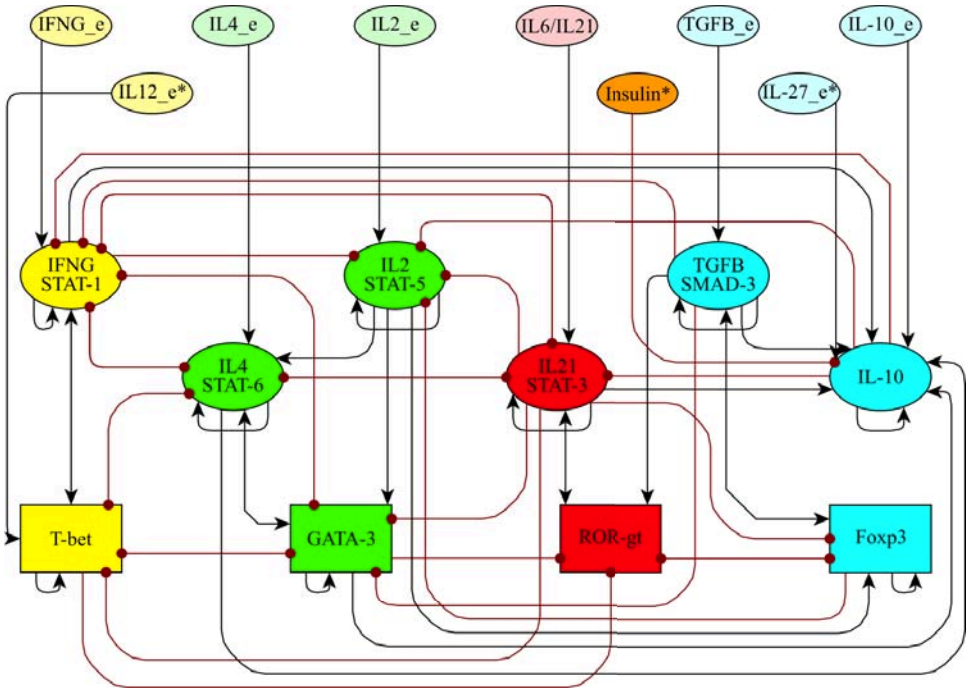


Fig 2. CD4+ T cell regulatory network. The network includes transcription factors (rectangles), intrinsic cytokines and their signaling pathways (ellipses), and extrinsic cytokines and insulin (ellipses). Node colors correspond to cell types in which each molecule is generally expressed (state = 1): Th1 (yellow), Th2 (green), Th17 (red), iTreg (blue), and insulin (orange). Activations between elements are represented with black arrows, and inhibitions with red dotted arrows. An * is used to indicate the new nodes considered in this network model with respect to that in (12).

Cytokines can be produced by the cell (intrinsic) or by other cells of the immune system (extrinsic). Such extrinsic cytokines constitute the micro-environment and have an important role in CD4+ T cell differentiation and plasticity. Extrinsic cytokines were considered as inputs of the system [Figure 1B]. To study the effect of the micro-environment we focused on six biologically relevant environments: pro-Th0 or resting, pro-Th1, pro-Th2, pro-Th17, pro-iTreg, and pro-IL10 [Table 1].

Environment	Cytokines	Active nodes
pro-Th0	no cytokines	None
pro-Th1	IFNγ, IL-12	IFNGe, IL12e
pro-Th2	IL-2, IL-4	IL2e, IL4e
pro-Th17	IL-21 (or IL-6), TGFβ	IL21e, TGFBe
pro-iTreg	IL-2, TGFβ	IL2e, TGFBe
pro-IL10	IL-10, 1L-27	IL10e, IL27e

Table 1: Environments of the CD4+ T cell regulatory network.

The stable states to which a regulatory network converge are called attractors, and can be interpreted as the expression profiles of the biological cell types (37, 38) [Figure 1B]. We labeled each attractor according to the active transcription factors and intrinsic cytokines [Table S2]. Th0, resting T cells, were defined as expressing no transcription factors or regulatory cytokines. Th1 was defined as having Tbet and IFNγ active, Th2 as GATA3 and IL-4 active and GATA3+ (a Th2-like cell type) as GATA3+IL4-. Th17 cells are characterized by the expression of RORγt and STAT3 signaling mediated by IL-6 or IL-21, all of which require the presence of TGF-βe. The iTreg type has Foxp3 and TGFβ, IL-10 or both, all of which require the presence of IL-2e. T regulatory Foxp3-independent cells feature IL-10 (IL10+), TGF-β (TGFβ+) or both (IL10+TGFβ+), without expressing Foxp3. Th1 regulatory cells (Th1R) express a regulatory cytokine and T-bet [46]. Th2 regulatory cells (Th2rR) express a regulatory cytokine and GATA3. The attractors obtained by the CD4 + T cell network correspond to configurations that are characteristic of: Th0, Th1, Th1R, Th2, GATA3+, Th2R, Th17, iTreg, TGFβ+IL10+, TGFβ+ and IL10+ CD4+ T cells [Figure S1] {Zhu2010, MartinezSanchez2015}.

Effect of insulin on CD4+ T cell differentiation

To simulate the effects of insulin, we obtained the attractors in the different micro-environments in the presence of basal levels (state of the "insulin" node to 0) or high levels (state of the "insulin" node to 1) of insulin [Figure 3]. To simulate the different environments we fixed the values of the input nodes according to each environment as listed in Table 1. Then, we determined and labeled the resulting attractors to obtain the predicted cell types under each environment and insulin condition [Figure 1B].

Our model shows that in effector polarizing environments with basal levels of insulin, like pro-Th1, pro-Th2 and pro-Th17, effector and regulatory cells coexist. In a pro-iTreg environment there is a coexistence of iTreg and Th17 cells. But in a pro-IL10 we see a strong polarization towards regulatory T cells and no effector CD4+ T cells. We observed that in the presence of high levels of insulin there is a marked decrease of the attractors that express IL-10 (Th1R, Th2R, and IL10+TGFβ+), and the remaining attractors tend to express TGFβ. There is an increase in the size of the basins of attraction of the Th17 and Th1 attractors. This is particularly notable in the pro-Th17 insulin environment, where the Th1R and IL10+TGFβ+ disappear, and the network converges to Th17. In the case of the Th1 attractor the increase in its basin size is smaller. Interestingly, this behavior corresponds to the observed increase in Th1 and Th17 and the decrease in Treg cells and IL-10 in obesity-associated chronic inflammation. The only exception to this pattern was observed under the pro-IL10 environment that remains unchanged by the level of insulin.

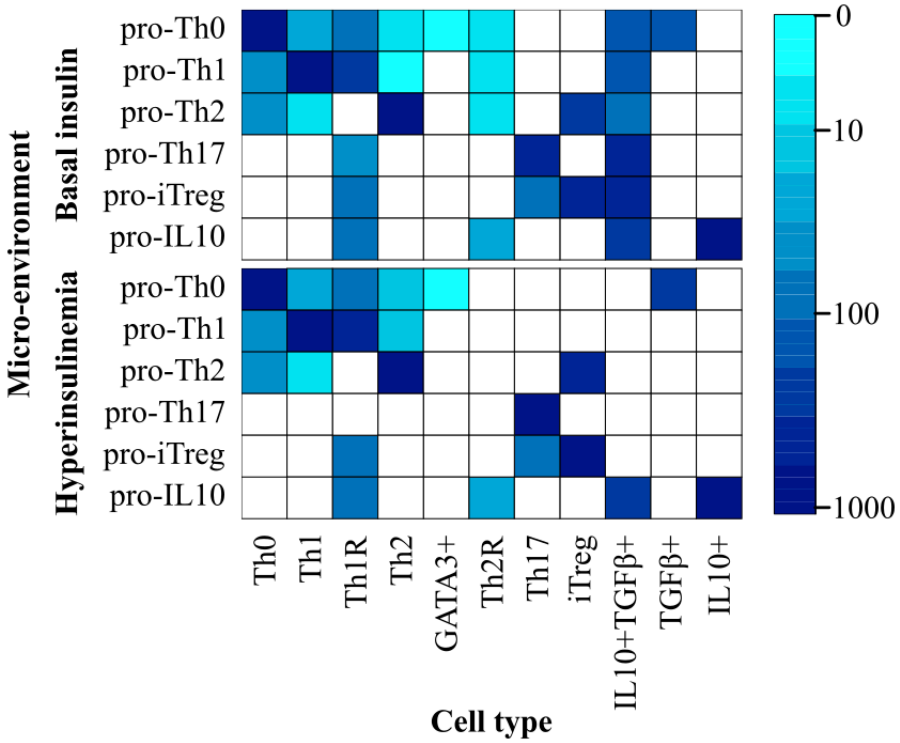


Fig 3. Effect of the micro-environment on CD4+ T cell differentiation. The values of the extrinsic signals of the TSRN were fixed according to different polarizing microenvironments. The color corresponds to the size of the basins of attraction on a logarithmic scale.

Effect of insulin on CD4+ T cell plasticity

CD4+ T cells are plastic and dynamically change from one type to others, depending on the microenvironment and transient perturbations or initial conditions. This implies that these cells configurations and behavior can be altered dynamically. The multistable Boolean network model used here is a useful tool to study CD4+ T cell plasticity (12) as well. To explore this, we transiently perturbed the attractors for each microenvironment (Table 1) under the constitutive presence of basal (0) and high levels of insulin (1). For each attractor, we transiently perturbed each node for one time step. Then, we returned the node to its original value and used the original logical rules, to recover the resulting attractor [Figure 1C]. We established that a labeled attractor was robust to a perturbation if it returned to the same configuration after such transient perturbation, or to one that corresponds to the same cell type, after a perturbation. When the system transitioned to an attractor that corresponds to a different cell type, we considered the original attractor to be plastic under transient perturbations.

Our results suggest that the effect of insulin on the differentiation and plasticity of CD4+ T cells depends on the cytokines that are present in the microenvironment [Figure 4, File S2]. In each microenvironment, without insulin, most of the transitions lead the system to the favored cell type, which tends to be the most stable one, as expected. But in these cases, other cell types also coexist in the environment, especially regulatory cell types, even though the attractors that characterize them are less stable. Under high levels of insulin, that simulates an acute hyperinsulinemia condition, the CD 4+ T plasticity patterns are altered. In general, the activation of insulin: (1) causes the loss of the regulatory attractors, particularly those that express IL-10-, reduces cell stability, and the number of transitions towards the original cell type. This is particularly notable in the pro-Th17 environment, where Th17 is the only possible attractor. In the case of the pro-iTreg environment, there is a coexistence of iTreg with Th17. This is caused by the extrinsic TGFβ. The role of TGFβ is bivalent, as it can induce both regulation and inflammation through iTreg and Th17 cells. In the case of TGFβ,

insulin shifts the equilibrium towards inflammatory cell types. The addition of insulin caused the loss of the IL10+TGFβ+ attractor, stabilized Th17, and reduced the stability of iTreg and Th1R. In the only case that this did not occur, was under the pro-IL10 environment, where a regulatory phenotype is attained independently of the insulin level, avoiding a pro-inflammatory condition even under the presence of hyperinsulinemia.

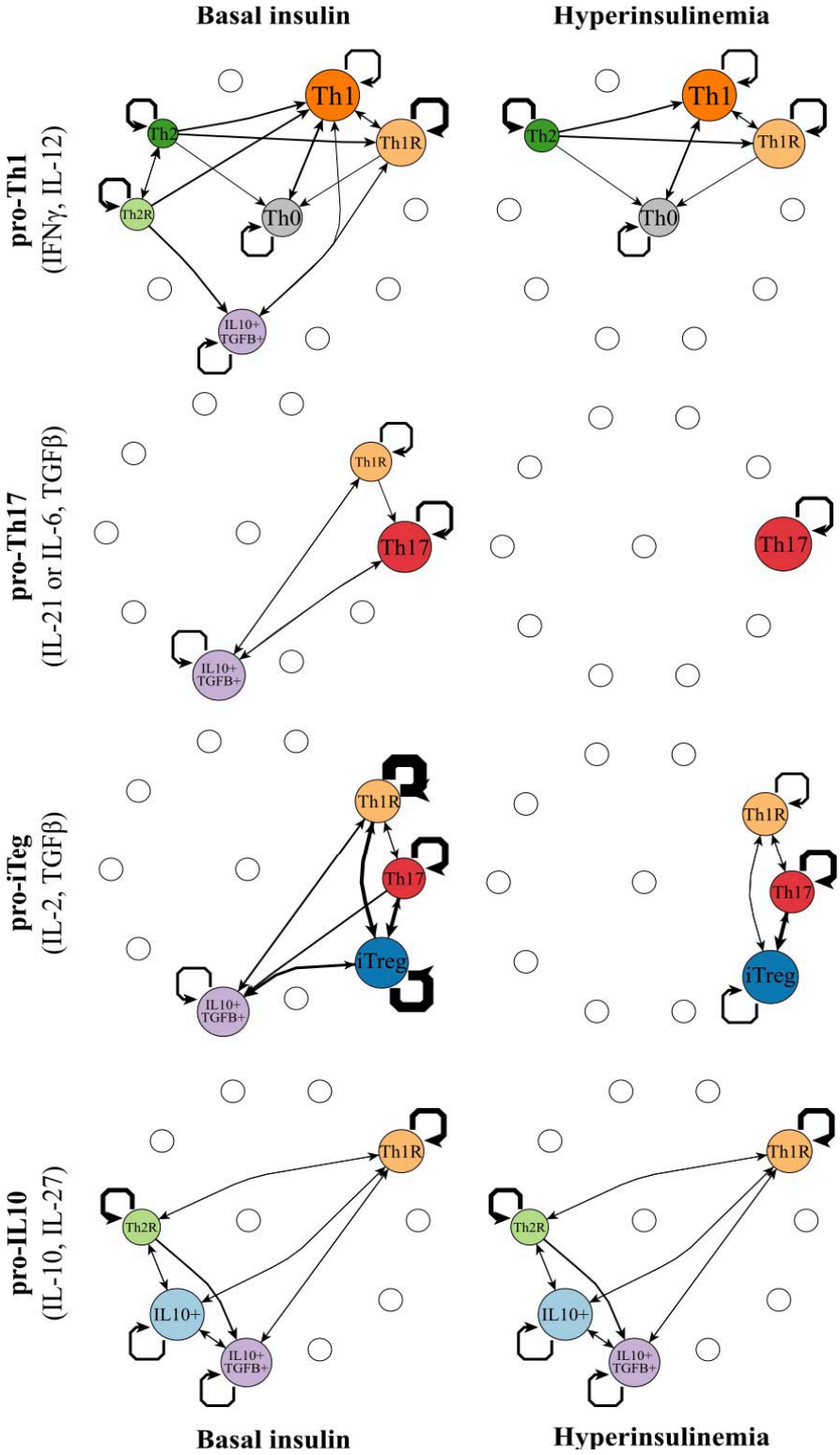


Fig 4. Cell fate map under different microenvironments. The values of the extrinsic signals of the CD4+ T regulatory network were fixed according to different polarizing microenvironments as listed in Table 1, and the resulting attractors were transiently perturbed for one time step. Nodes correspond to cell types, node size is proportional to the number of configurations in a basin of attraction. Edges represent transitions from one cell type to another, their width represents the number of times the transition occurred, self-loops correspond to perturbations in which the network returned to the original cell type. The following micro-environments were studied:: (A) pro-Th1, (B) pro-Th1+ Insulin, (C) pro-Th17, (D) pro-Th17 + Insulin, (E) pro-iTreg, (F) pro-iTreg + Insulin, (G) pro-IL10 (H) pro-IL10.

The role of IL10 on CD4+ T cell plasticity alterations under normal and hyperinsulinemic conditions

We assessed how many transitions among attractors were caused by transient perturbations of insulin and IL10 under normal and hyperinsulinemic conditions. On average perturbations of any node caused transitions to new cell types in 38% of the cases. But the number of transitions between cell types varied according to the node and the microenvironment. The transient increase of insulin caused transitions towards inflammatory or TGFB producing cell types under basal insulin level, while, as expected, under hyperinsulinemia the transient activation of insulin did not cause any further transitions. The attractors of the pro-Th17 environment with basal levels of insulin were very sensitive to perturbations in the insulin node, while the attractors found in the pro-iTreg and the pro-IL10 environment were robust to this perturbations. The transient activation of IL-10 caused transitions towards regulatory cell types. The attractors of the pro-Th1, pro-Th17 with basal levels of insulin and the pro-iTreg with high level of insulin environments were, on the other hand, very sensitive to transient perturbations of IL10. In environments with basal levels of insulin, the transient activation of IL-10 caused some transitions towards Th1 and Th2 cell types in pro-Th1 and pro-Th2 environments, respectively. In these cases, the transient activation of IL-10 was sufficient to destabilize the attractor but not to shift the network towards a regulatory cell type.

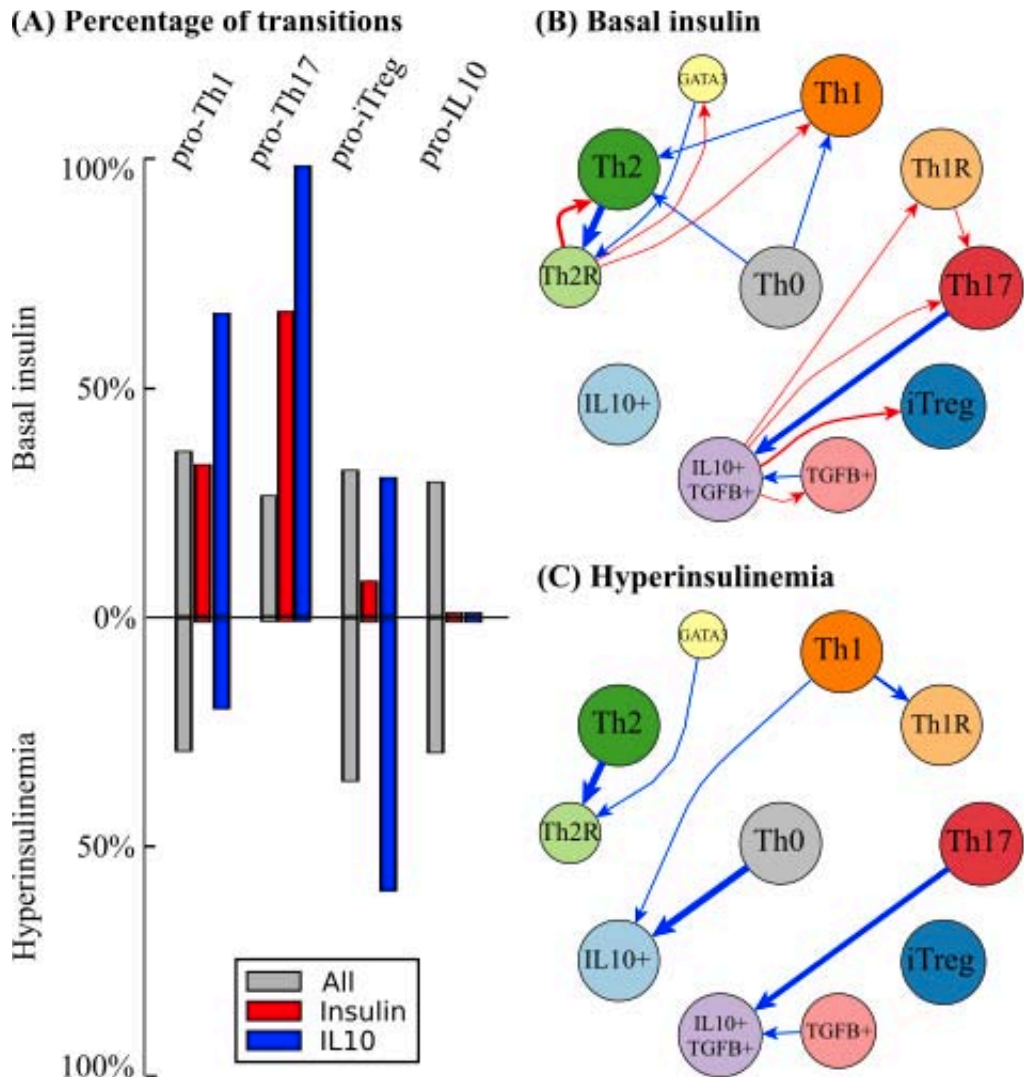


Fig 5. Transitions between cell types caused by the transient activation of the insulin and the IL-10 nodes. (A) Percentage of transitions between attractors in response to transient perturbations of: all nodes (grey); insulin (red) or IL-10 nodes (blue) under basal and high levels of insulin. (B, C) Transitions between cell types caused by the transient activation of: insulin (red) or IL-10 nodes (blue) under basal (B) and high levels (C) of insulin.

Discussion

The theoretical simulation study presented here suggests that the impact of hyperinsulinemia on the inflammatory response (14), is mediated by the multistable dynamic GRN in (12). Overall, our simulation study provides a system-level platform to explain the relationship between hyperinsulinemia and altered proportions of T regulatory cells that have been observed in adipose tissue (3, 32-34, 36). It also highlights and provides a dynamic explanation to the different roles of TGFβ and IL-10 (25-28).

The model shows that in pro-Th1, pro-Th2, pro-Th17 and pro-iTreg microenvironments, effector and regulatory cells coexist. This pattern is observed in any disease, where it is common to observe cells from different subsets, even if a specific one is over-represented(39). Moreover, the simulations predict that different types of regulatory cells will predominate depending on the environment, being especially important to distinguish Foxp3+ and Fop3- regulatory T cells. Future experiments should consider that CD4+ T cells are highly heterogeneous, phenotypically plastic and

sensitive to the microenvironment. A CD4+ T cell can express markers for more than one cell type at the same time, and its expression patterns can change over time, especially for regulatory T cells. It is necessary to measure the expression of Foxp3, IL-10, and TGFβ to systemically distinguish Treg (Foxp3+CD25high), Foxp3-IL10+, Foxp3-TGFβ+ and Th1R and Th2R hybrid cell types. Distinguishing these cell types will be necessary to understand the different roles that they play in obesity-associated chronic inflammation. Future assays should also consider multiple transcription factors and cytokines, carefully separate CD4+ T cell populations and compare their behaviors in different tissues.

Our simulation results recovered the altered CD4+ T cell populations that have been observed in murine models and humans during obesity-associated chronic inflammation (3, 32-34, 36). In the presence of hyperinsulinemia, increased proportions of Th1 and Th17 cells and decreased proportions of regulatory T cells are observed (3, 33, 34). Specifically, in a pro-Th17 environment, the presence of insulin predicts a complete shift towards Th17 cells. In contrast, in a pro-Th1 environment the Th1 attractor alteration is less dramatic than the alterations observed in vivo, probably because of the involvement of macrophages in the real condition, that are not considered in the simulation model of this study (3, 33, 34).

The model also provides an explanation to some paradoxical behaviors observed in CD4+ T regulatory cell populations during obesity-associated chronic inflammation. TGFβ can promote both inflammatory Th17 cells and regulatory Tregs, and transitions between both subsets have been observed (3, 33, 34, 40, 41). The model gives a mechanistic explanation to the fact that Th17 cells and iTregs are closely related and that Th17 cells can be observed sometimes during the iTreg response. TGFβ is necessary for the differentiation of both subsets, and transient signaling via the STAT3 pathway may be enough to shift some cells towards Th17, as the model shows. In obesity, Tregs expression profiles are similar to inflammatory T cells (32). Transfer and depletion of adipose Treg cells have been reported to both, improve or worsen insulin sensitivity, depending on the model and the population studied (33, 35, 36). Such apparently paradoxical behaviors can be explained by the relationship between TGFβ and IL-10 in the context of the dynamic regulatory network model used here. Under hyperinsulinemia, Th17 cells become more stable while IL10+ cells are lost. The remaining regulatory cells express TGFβ that is involved in Th17 differentiation, while insulin alters iTregs stability. In this way, the model predicts that hyperinsulinemic inflammatory environments, specially under pro-Th17 conditions, T regulatory cells are lost and the rest become unstable. In contrast, a pro-IL10 environment can induce regulatory T cells, regardless of the level of insulin in the environment. Nonetheless, while this pro-regulatory environment might decrease inflammation, it may have adverse effects as inflammation is relevant for the function of adipose tissue (42).

The model predicts that the transitions between cell types vary depending on the microenvironment and the perturbed node. Transient activation of insulin is sufficient to cause transitions towards inflammatory or TGFB+ cells, while transient activation of IL10 is sufficient to cause transitions towards regulatory cells. The stability of the different cell types will also vary depending on the microenvironment and the perturbation. We predict that the cells in a pro-Th17 environment are more sensitive to transient increases in insulin, while the cells in a pro-iTreg and pro-IL10 environments are more stable under this perturbation.

The model used here considers a minimum regulatory network underlying CD4+ T cell differentiation and plasticity under hyperinsulinemia, but it still lacks other cells and signals that are fundamental to fully understand obesity-associated chronic inflammation. For example, since the network used here is a minimal model, it ignores cytokines such as IL-1 and TNFα, the role of sexual hormones, and additional cell types such as adipocytes and macrophages, that play important roles during obesity-associated chronic inflammation (15-17). The model is restricted to assess the role of insulin on the differentiation dynamics of an activated CD4+ T cell in VAT, but still lacks the regulation

of the TCR signaling pathway and the contrasting metabolism among effector, resting and regulatory conditions.

Furthermore, future efforts should consider continuous versions of the model to enable simulations of the strength and length of the signals in the dynamics of the immune system. Such simulations may be useful to assess different treatments of metabolic disorders and chronic inflammation, as well as the actual timing and progression of the obese inflammatory response. The model used here, still simplifies the microenvironment, that is more complex in vivo. For example, it is interesting to asses how the small initial signals that occur in response to nutrient overload, eventually give rise to significant alterations associated to obesity-associated chronic inflammation (15). Further studies of the effect of transient signals in a continuous version of the minimum and extended CD4+ T cell regulatory network, will likely yield important insights concerning such temporal patterns. Such system-level approach will be also useful for toxicological studies, and for providing predictions concerning the biological impact of drugs, assessing therapeutic targets or secondary effects.

Materials and Methods

Logical modeling formalism: Boolean networks

A Boolean network is composed of nodes that represent the system´s molecular components (i.e., cytokines, signaling pathways or transcription factors) and edges, that represent the interactions between nodes. The value of the nodes can be associated with a discrete variable denoting its current functional level of activity: if the node is functional its value is 1, and if it is not functional it is 0. The value of a node $x_i(t+1)$ depends on the value of its input nodes or regulators, this can be expressed with a Boolean function:

$$x_i(t+1)=f(x_1(t), x_2(t), \dots , x_n(t))$$

For the Th+insulin network, the Boolean functions were defined based on available T CD4+ differentiation models(10-12) and experimental data for the reported interactions among a network more than 90 nodes [Table S1]. The network was then simplified as (43) and GINSIM(44) . The resulting network has 19 nodes and 54 interactions.

Dynamic analysis

The state of the network X can be represented by a vector that specifies the value of all the nodes of the system. The state of the network will change over time depending on the Boolean functions associated with each node. When the values of a state vector X at $t+1$ are the same as those at time $t+\tau$, the system has reached an attractor X^*:

$$X^*(t) = X(t+\tau), \qquad \tau >= 1.$$

An attractor can be interpreted as a stable expression phenotype of a cell or cell type (45). All the states that lead to a solution X^* constitute the basin of attraction of such an attractor. We determined the stable states and basins of attraction of the network using GINSIM (44) and BoolNet(46).

Labeling

Attractors were labeled depending on the expression of both the master transcription factors and cytokines. Labeling was automatized using BoolNetPerturb (47).

Perturbations

To study the plasticity in response to perturbations we used BoolNetPerturb (47). First, we took all the attractors in each microenvironment, and systematically perturbed the value of the node for a time step, fixing the value of the target node during the corresponding time period. As the perturbation was transient, after a time step the node returns to its original function or -in the case of the inputs- to is

original value. Finally, we reported the attractor that was reached after the perturbation. If the network returned to an attractor with the same label as the original attractor we said it was stable to that specific perturbation, if the network return to a different labeled attractor we said there had been a transition from one cell type to an other.

References

1. S. Winer and D. a Winer, “The adaptive immune system as a fundamental regulator of adipose tissue inflammation and insulin resistance,” Immunol. Cell Biol., vol. 90, no. 8, pp. 755–762, 2012.
2. J. M. Han and M. K. Levings, “Immune regulation in obesity-associated adipose inflammation.,” J. Immunol., vol. 191, no. 2, pp. 527–32, 2013.
3. V. A. Gerriets and N. J. MacIver, “Role of T cells in malnutrition and obesity.,” Front. Immunol., vol. 5, no. August, p. 379, 2014.
4. M. Hiriart, M. Velasco, C. Larque, and C. M. Diaz-Garcia, “Metabolic syndrome and ionic channels in pancreatic beta cells,” Vitam. Horm., vol. 95, pp. 87–114, 2014.
5. E. Balleza, E. R. Alvarez-Buylla, A. Chaos, S. Kauffman, I. Shmulevich, and M. Aldana, “Critical dynamics in genetic regulatory networks: examples from four kingdoms.,” PLoS One, vol. 3, no. 6, p. e2456, Jan. 2008.
6. S. Huang, “Systems biology of stem cells: three useful perspectives to help overcome the paradigm of linear pathways.,” Philos. Trans. R. Soc. Lond. B. Biol. Sci., vol. 366, no. 1575, pp. 2247–59, Aug. 2011.
7. P. Creixell, J. Reimand, S. Haider, G. Wu, T. Shibata, M. Vazquez, V. Mustonen, A. Gonzalez-Perez, J. Pearson, C. Sander, B. J. Raphael, D. S. Marks, B. F. F. Ouellette, A. Valencia, G. D. Bader, P. C. Boutros, J. M. Stuart, R. Linding, N. Lopez-Bigas, and L. D. Stein, “Pathway and network analysis of cancer genomes,” Nat. Methods, vol. 12, no. 7, pp. 615–621, 2015.
8. S. M. Hill, L. M. Heiser, T. Cokelaer, M. Unger, N. K. Nesser, D. E. Carlin, Y. Zhang, A. Sokolov, E. O. Paull, C. K. Wong, K. Graim, A. Bivol, H. Wang, F. Zhu, B. Afsari, L. V Danilova, A. V Favorov, W. S. Lee, D. Taylor, C. W. Hu, B. L. Long, D. P. Noren, A. J. Bisberg, B. Afsari, R. Al-Ouran, B. Anton, T. Arodz, O. A. Sichani, N. Bagheri, N. Berlow, A. J. Bisberg, A. Bivol, A. Bohler, J. Bonet, R. Bonneau, G. Budak, R. Bunescu, M. Caglar, B. Cai, C. Cai, D. E. Carlin, A. Carlon, L. Chen, M. F. Ciaccio, T. Cokelaer, G. Cooper, C. J. Creighton, S.-M.-H. Daneshmand, A. de la Fuente, B. Di Camillo, L. V Danilova, J. Dutta-Moscato, K. Emmett, C. Evelo, M.-K. H. Fassia, A. V Favorov, E. J. Fertig, J. D. Finkle, F. Finotello, S. Friend, X. Gao, J. Gao, J. Garcia-Garcia, S. Ghosh, A. Giaretta, K. Graim, J. W. Gray, R. Großeholz, Y. Guan, J. Guinney, C. Hafemeister, O. Hahn, S. Haider, T. Hase, L. M. Heiser, S. M. Hill, J. Hodgson, B. Hoff, C. H. Hsu, C. W. Hu, Y. Hu, X. Huang, M. Jalili, X. Jiang, T. Kacprowski, L. Kaderali, M. Kang, V. Kannan, M. Kellen, K. Kikuchi, D.-C. Kim, H. Kitano, B. Knapp, G. Komatsoulis, H. Koeppl, A. Krämer, M. B. Kursa, M. Kutmon, W. S. Lee, Y. Li, X. Liang, Z. Liu, Y. Liu, B. L. Long, S. Lu, X. Lu, M. Manfrini, M. R. A. Matos, D. Meerzaman, G. B. Mills, W. Min, S. Mukherjee, C. L. Müller, R. E. Neapolitan, N. K. Nesser, D. P. Noren, T. Norman, B. Oliva, S. O. Opiyo, R. Pal, A. Palinkas, E. O. Paull, J. Planas-Iglesias, D. Poglayen, A. A. Qutub, J. Saez-Rodriguez, F. Sambo, T. Sanavia, A. Sharifi-Zarchi, J. Slawek, A. Sokolov, M. Song, P. T. Spellman, A. Streck, G. Stolovitzky, S. Strunz, J. M. Stuart, D. Taylor, J. Tegnér, K. Thobe, G. M. Toffolo, E. Trifoglio, M. Unger, Q. Wan, H. Wang, L. Welch, C. K. Wong, J. J. Wu, A. Y. Xue, R. Yamanaka, C. Yan, S. Zairis, M. Zengerling, H. Zenil, S. Zhang, Y. Zhang, F. Zhu, Z. Zi, G. B. Mills, J. W. Gray, M. Kellen, T. Norman, S. Friend, A. A. Qutub, E. J. Fertig, Y. Guan, M. Song, J. M. Stuart, P. T. Spellman, H. Koeppl, G. Stolovitzky, J. Saez-Rodriguez, and S.

Mukherjee, “Inferring causal molecular networks: empirical assessment through a community-based effort,” Nat. Methods, no. February 2015, 2016.

9. J. M. Olefsky and C. K. Glass, “Macrophages, inflammation, and insulin resistance.,” Annu. Rev. Physiol., vol. 72, pp. 219–46, Jan. 2010.
10. A. Carbo, R. Hontecillas, B. Kronsteiner, M. Viladomiu, M. Pedragosa, P. Lu, C. W. Philipson, S. Hoops, M. Marathe, S. Eubank, K. Bisset, K. Wendelsdorf, A. Jarrah, Y. Mei, and J. Bassaganya-Riera, “Systems modeling of molecular mechanisms controlling cytokine-driven CD4+ T cell differentiation and phenotype plasticity.,” PLoS Comput. Biol., vol. 9, no. 4, p. e1003027, Apr. 2013.
11. W. Abou-Jaoudé, P. T. Monteiro, A. Naldi, M. Grandclaudon, V. Soumelis, C. Chaouiya, and D. Thieffry, “Model checking to assess T-helper cell plasticity.,” Front. Bioeng. Biotechnol., vol. 2, p. 86, Jan. 2014.
12. M. E. Martinez-Sanchez, L. Mendoza, C. Villarreal, and E. R. Alvarez-Buylla, “A Minimal Regulatory Network of Extrinsic and Intrinsic Factors Recovers Observed Patterns of CD4+ T Cell Differentiation and Plasticity.,” PLoS Comput. Biol., vol. 11, no. 6, p. e1004324, Jul. 2015.
13. N. J. MacIver, R. D. Michalek, and J. C. Rathmell, “Metabolic Regulation of T Lymphocytes.,” Annu. Rev. Immunol., vol. 31, no. December 2012, pp. 259–283, Jan. 2013.
14. J. M. Han, S. J. Patterson, M. Speck, J. a. Ehses, and M. K. Levings, “Insulin inhibits IL-10-mediated regulatory T cell function: implications for obesity.,” J. Immunol., vol. 192, no. 2, pp. 623–9, Jan. 2014.
15. M. F. Gregor and G. S. Hotamisligil, “Inflammatory mechanisms in obesity.,” Annu. Rev. Immunol., vol. 29, pp. 415–45, Apr. 2011.
16. M. Hiriart, C. L. , Myrian Velasco, Carlos Manlio Diaz-Garcia, A. ´, Carmen Sa ´nchez-Soto, J. P. C. Albarado-Iba˜ez, and G. ´n ´vez-Maldonado, Alicia Toledo and Neivys Garcı´a-DelgadoKanakis, “Pancreatic Beta Cells in Metabolic Syndrome,” in Islets of Langerhans SE - 50, 2014, pp. 1–29.
17. J. Hirosumi, G. Tuncman, L. Chang, C. Z. Görgün, K. T. Uysal, K. Maeda, M. Karin, and G. S. Hotamisligil, “A central role for JNK in obesity and insulin resistance.,” Nature, vol. 420, no. 6913, pp. 333–6, Dec. 2002.
18. C. T. Kelly, J. Mansoor, G. L. Dohm, W. H. H. Chapman, J. R. Pender, and W. J. Pories, “Hyperinsulinemic syndrome: The metabolic syndrome is broader than you think,” Surg. (United States), vol. 156, no. 2, pp. 405–411, 2014.
19. J. Zhu, H. Yamane, W. E. W. Paul, and Z. J. Y. H. P. WE, “Differentiation of effector CD4 T cell populations,” Annu. Rev. Immunol., vol. 28, no. 1, pp. 445–89, Jan. 2010.
20. T. Hong, J. Xing, L. Li, and J. J. Tyson, “A simple theoretical framework for understanding heterogeneous differentiation of CD4+ T cells.,” BMC Syst. Biol., vol. 6, no. 1, p. 66, Jan. 2012.
21. S. Huang, “Hybrid T-Helper Cells: Stabilizing the Moderate Center in a Polarized System,” PLoS Biol., vol. 11, no. 8, pp. 1–5, 2013.
22. K. K. M. K. M. Murphy, B. Stockinger, and A. Manuscript, “Effector T cell plasticity: flexibility in the face of changing circumstances,” Nat. Immunol., vol. 11, no. 8, pp. 674–680, Aug. 2010.
23. S. Sakaguchi, D. a a Vignali, A. Y. Rudensky, R. E. Niec, and H. Waldmann, “The plasticity and stability of regulatory T cells.,” Nature reviews. Immunology, vol. 13, no. 6. pp. 461–7, 2013.
24. P. We, “Fundamental Immunology 6th ed.” Lippincott Williams & Wilkins, Reading, Massachusetts, 2008.
25. F. Fantini MC Becker C, “Cutting edge: TGF-β induces a regulatory phenotype in CD4+CD25− T cells through Foxp3 induction and down-regulation of Smad7,” J Immunol, vol. 172, pp. 5149–5153, 2004.

26. B. Veldhoen M Hocking RJ, M. Veldhoen, R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger, “TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells,” Immunity, vol. 24, no. 2, pp. 179–189, Feb. 2006.
27. M. a Travis and D. Sheppard, “TGF-β activation and function in immunity.,” Annu. Rev. Immunol., vol. 32, no. November 2013, pp. 51–82, 2014.
28. A. Howes, P. Stimpson, P. Redford, L. Gabrysova, and A. O’Garra, “Interleukin-10: Cytokines in Anti-inflammation and Tolerance,” in Cytokine Frontiers: Regulation of Immune Responses in Health and Disease, vol. 6, 2014, pp. 327–352.
29. M. a Koch, G. Tucker-Heard, N. R. Perdue, J. R. Killebrew, K. B. Urdahl, D. J. Campbell, “The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation.,” Nat. Immunol., vol. 10, no. 6, pp. 595–602, Jun. 2009.
30. M. J. Barnes and F. Powrie, “Hybrid T reg cells : steel frames and plastic exteriors,” Immunity, vol. 10, no. 6, pp. 11–12, 2009.
31. Y. Wang, M. A. Su, and Y. Y. Wan, “An essential role of the transcription factor GATA-3 for the function of regulatory T cells.,” Immunity, vol. 35, no. 3, pp. 337–348, Sep. 2011.
32. M. Feuerer, L. Herrero, D. Cipolletta, A. Naaz, J. Wong, J. Lee, A. Goldfine, C. Benoist, S. Shoelson, and D. Mathis, “Fat Treg cells: a liaison between the immune and metabolic systems,” Nat. Med., vol. 15, no. 8, pp. 930–939, 2009.
33. O. Osborn and J. M. Olefsky, “The cellular and signaling networks linking the immune system and metabolism in disease,” Nat. Med., vol. 18, no. 3, pp. 363–374, 2012.
34. T. McLaughlin, L. F. Liu, C. Lamendola, L. Shen, J. Morton, H. Rivas, D. Winer, L. Tolentino, O. Choi, H. Zhang, M. H. Y. Chng, and E. Engleman, “T-cell profile in adipose tissue is associated with insulin resistance and systemic inflammation in humans,” Arterioscler. Thromb. Vasc. Biol., vol. 34, no. 12, pp. 2632–2636, 2014.
35. M. Feuerer, L. Herrero, D. Cipolletta, A. Naaz, J. Wong, A. Nayer, J. Lee, A. B. Goldfine, C. Benoist, S. Shoelson, and D. Mathis, “Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters.,” Nat. Med., vol. 15, no. 8, pp. 930–9, Aug. 2009.
36. S. P. Bapat, J. Myoung Suh, S. Fang, S. Liu, Y. Zhang, A. Cheng, C. Zhou, Y. Liang, M. LeBlanc, C. Liddle, A. R. Atkins, R. T. Yu, M. Downes, R. M. Evans, and Y. Zheng, “Depletion of fat-resident Treg cells prevents age-associated insulin resistance,” Nature, 2015.
37. E. Azpeitia, M. Benítez, P. Padilla-Longoria, C. Espinosa-Soto, and E. R. Alvarez-Buylla, “Dynamic network-based epistasis analysis: boolean examples.,” Front. Plant Sci., vol. 2, p. 92, Jan. 2011.
38. R. Albert and J. Thakar, “Boolean modeling: a logic-based dynamic approach for understanding signaling and regulatory networks and for making useful predictions,” Wiley Interdiscip. Rev. Syst. Biol. Med., vol. 6, no. 5, pp. 353–369, Sep. 2014.
39. M. DuPage and J. A. Bluestone, “Harnessing the plasticity of CD4+ T cells to treat immune-mediated disease,” Nat. Rev. Immunol., vol. 16, no. 3, pp. 149–163, Feb. 2016.
40. Y. K. Lee, R. Mukasa, R. D. Hatton, and C. T. Weaver, “Developmental plasticity of Th17 and Treg cells.,” Curr. Opin. Immunol., vol. 21, no. 3, pp. 274–80, Jun. 2009.
41. M. Kleinewietfeld and D. A. Hafler, “The plasticity of human Treg and Th17 cells and its role in autoimmunity.,” Semin. Immunol., vol. 25, no. 4, pp. 305–12, Nov. 2013.
42. I. Wernstedt Asterholm, C. Tao, T. S. Morley, Q. A. Wang, F. Delgado-Lopez, Z. V Wang, and P. E. Scherer, “Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling.,” Cell Metab., vol. 20, no. 1, pp. 103–18, Jul. 2014.
43. J. Davila-Velderrain, C. Villarreal, and E. R. Alvarez-Buylla, “Reshaping the epigenetic landscape during early flower development: induction of attractor transitions by relative differences in gene decay rates.,” BMC Syst. Biol., vol. 9, no. 1, p. 20, 2015.

44. A. Naldi, D. Berenguier, A. Fauré, F. Lopez, D. Thieffry, and C. Chaouiya, “Logical modelling of regulatory networks with GINsim 2.3.,” Biosystems., vol. 97, no. 2, pp. 134–9, Aug. 2009.
45. S. A. Kauffman, “Metabolic stability and epigenesis in randomly constructed genetic nets,” J. Theor. Biol., vol. 22, no. 3, pp. 437–467, Mar. 1969.
46. C. Müssel, M. Hopfensitz, and H. A. Kestler, “BoolNet-an R package for generation, reconstruction and analysis of Boolean networks,” Bioinformatics, vol. 26, no. 10, pp. 1378–1380, 2010.
47. M. E. Martinez-Sanchez. BoolNet Perturb, GitHub repository: https://github.com/mar-esther23/boolnet-perturb. 2015
48. Chelliah V et al. BioModels: ten-year anniversary. Nucl. Acids Res. 2015, 43(Database issue):D542-8

Acknowledgments

This work is presented in partial fulfillment towards Mariana Martínez-Sanchez doctoral degree in the program “Doctorado en Ciencias Biomédicas, de la Universidad Nacional Autónoma de México”. We acknowledge the support Alejandro Frank and the “Centro de Ciencias de la Complejidad (C3), de la Universidad Nacional Autónoma de México”. We also acknowledge Diana Romo for her help with many logistical tasks and E. Roces de Álvarez-Buylla for her comments about this work. **Funding**: ERAB received funding from: CONACYT: 240180, 180380, 152649 and UNAM-DGAPA-PAPIIT: IN 203214, IN203814, IN211516. MI received funding from: PAPIIT IV100116. **Author contributions**: Conceived and designed the experiments: ERAB MEMS. Performed the experiments: MEMS. Analyzed the data: ERAB MEMS MH. Contributed reagents/materials/analysis tools: MEMS. Wrote the paper: MEMS ERAB MH. **Competing interests**: The authors declare that they have no competing interests. **Data and materials availability**: This model was deposited in BioModels and assigned the identifier MODEL1606020000. The code and tutorials for the robustness and plasticity analysis are available at https://github.com/mar-esther23/boolnet-perturb.

Capítulo 4

The combination of the functionalities of feedback circuits is determinant for the number and size of attractors of molecular networks

SCIENTIFIC REPORTS

OPEN

The combination of the functionalities of feedback circuits is determinant for the attractors' number and size in pathway-like Boolean networks

Received: 27 May 2016
Accepted: 04 January 2017
Published: xx xx xxxx

Eugenio Azpeitia[1], Stalin Muñoz[2,*], Daniel González-Tokman[3,*], Mariana Esther Martínez-Sánchez[4,5,*], Nathan Weinstein[6,*], Aurélien Naldi[7], Elena R. Álvarez-Buylla[5,8], David A. Rosenblueth[2] & Luis Mendoza[9]

Molecular regulation was initially assumed to follow both a unidirectional and a hierarchical organization forming pathways. Regulatory processes, however, form highly interlinked networks with non-hierarchical and non-unidirectional structures that contain statistically overrepresented circuits or motifs. Here, we analyze the behavior of pathways containing non-unidirectional (i.e. bidirectional) and non-hierarchical interactions that create motifs. In comparison with unidirectional and hierarchical pathways, our pathways have a high diversity of behaviors, characterized by the size and number of attractors. Motifs have been studied individually showing that feedback circuit motifs regulate the number and size of attractors. It is less clear what happens in molecular networks that usually contain multiple feedbacks. Here, we find that the way feedback circuits couple to each other (i.e., the combination of the functionalities of feedback circuits) regulate both the number and size of the attractors. We show that the different sets of expected results of epistasis analysis (a method to infer regulatory interactions) are produced by many non-hierarchical and non-unidirectional structures. Thus, these structures cannot be correctly inferred by epistasis analysis. Finally, we show that the combinations of functionalities, combined with other network properties, allow for a better characterization of regulatory structures.

Early approaches considered that molecular regulation is composed of hierarchical and unidirectional interactions, where "upstream" molecules regulate "downstream" molecules, but molecules are not regulated by molecules at the same or lower levels (molecules usually represent genes and gene products[1]). Unidirectional and hierarchical interactions form pathways, comprised by an input, internal molecules, and an output. Pathway dynamics (i.e., how the components in the pathway are activated and inhibited in time), follow a sequential order of regulatory events going from the input to the output through the internal molecules. Even though pathway dynamics seem to be an inherent property of molecular regulation[2,3], molecular regulation is usually complex, forming highly inter-connected networks that are neither unidirectional nor hierarchical[4–6]. Our objective is to

[1]INRIA project-team Virtual Plants, joint with CIRAD and INRA, Montpellier Cedex 5, France. [2]Instituto de Investigaciones en Matemáticas Aplicadas y en Sistemas, Universidad Nacional Autónoma de México, Apdo. 20-126, 01000 México, D.F., México. [3]CONACYT, Instituto de Ecología, A. C., Antiguo camino a Coatepec 351, El Haya, 91070 Xalapa, Veracruz, México. [4]Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, México. [5]Instituto de Ecología, Universidad Nacional Autónoma de México, México. [6]ABACUs-Centro de Matemáticas Aplicadas y Cómputo de Alto Rendimiento, Departamento de Matemáticas, Centro de Investigación y de Estudios Avanzados CINVESTAV-IPN, Carretera México-Toluca Km 38.5, La Marquesa, Ocoyoacac, Estado de México, 52740 México. [7]DIMNP UMR CNRS 5235, Université de Montpellier, France. [8]Centro de Ciencias de la Complejidad, Universidad Nacional Autónoma de México, México. [9]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to E.A. (email: eugenio.azpeitia-espinosa@inria.fr)

systematically study the effect of including non-hierarchical and non-unidirectional (i.e. bidirectional) interactions within pathways.

Adding interactions within a pathway modifies the pathway structure (i.e., the interaction graph describing the regulatory interactions between the molecules), allowing for the appearance of regulatory motifs. Motifs are statistically overrepresented regulatory interactions found in molecular networks[5]. Among the motifs, circular chains of oriented interactions known as feedback circuits, are specially relevant, because they regulate both the number and size of the attractors[7,8]. Attractors are stationary network states that represent biologically meaningful properties[9], such as cell identity[10,11]. Positive feedback circuits are necessary to have multiple attractors and negative feedback circuits are necessary to produce cyclic attractors[12,13]. Moreover, the maximum possible number of attractors is regulated by positive feedback circuits[14]. It is not completely clear, however, how the precise size and number of attractors are regulated within molecular networks where many feedback circuits are present[15–17]. In this work, we focus on the effect that adding non-hierarchical and non-unidirectional interactions has over the size and the number of attractors. In particular, we study how multiple feedback circuits, that result from the added interactions, regulate the size and number of attractors.

The study of the properties of non-hierarchical and non-unidirectional networks is fundamental to detect and solve limitations of traditional analyses. In particular, experimental research at small scales commonly uses traditional analyses that rely on the hierarchical and unidirectional assumptions. For example, assuming that regulatory interactions are hierarchical and unidirectional, epistasis analysis, as described in refs 1,18,19, can distinguish between different pathway structures by interpreting and organizing epistatic interactions (sensu Bateson[20]). The presence of non-unidirectional and non-hierarchical interactions, however, can produce incomplete or even wrong inferences when using epistasis analysis[21]. Here, we look for useful traits to distinguish between non-hierarchical and non-unidirectional regulatory structures that could improve inference methods, such as epistasis analysis.

In this work, we use synchronous Boolean networks to do a comprehensive analysis of Pathway-like networks (PLNs) that are non-hierarchical and non-unidirectional versions of pathways. We focus on the dynamical properties of PLNs, represented by both the number and the size of attractors. We show that PLNs have a large dynamical diversity in comparison with pathways. We confirm that feedback circuits are important regulators of the size and the number of attractors in a network. Then, we show for the first time, as far as we know, that the precise size and number of attractors in networks with multiple feedback circuits is in large part determined by the combination of the functionalities of feedback circuits. This combination refers to the network states where the feedback circuits in a network have an actual effect over the network dynamics. Then, we show that there are a vast number of PLNs producing the same set of attractors, hindering a correct inference of PLNs structures with methods such as epistasis analysis. Interestingly, PLNs producing the same set of attractors also have remarkably similar sets of combinations of functionalities. Thus, we explore how to use the combination of functionalities combined with both the network dynamic and structure for the characterization of PLNs. Our results show that PLNs with the same structure and with the same combination of functionality form small regions with dynamically distinguishable properties. Thus, the study of such regions could facilitate the inference of such networks.

Methods

Boolean networks. In this work, we use the Boolean formalism to model molecular networks for the following reasons. (1) Because of their simplicity, Boolean networks are well suited to perform analyses in a large number of networks, without needing to deal with problems such as parameter estimation. (2) Despite their simplicity, Boolean networks obtain biologically meaningful results (e.g., refs 10,11,22–24). (3) Feedback circuits are fundamental for this work, and feedback circuit functionality is well studied in Boolean networks[25,26]. (4) Here we will focus on the size and number of attractors, and it has been proven that in Boolean networks positive and negative feedback circuits are a necessary condition for multistability and oscillations, respectively[13,27]. (5) We will use epistasis analysis, as described in refs 1,18,19, that assumes that the molecules behave as Boolean variables. Hence Boolean networks are a natural and simple extension of epistasis analysis.

Molecular networks have variables representing the molecules included in the network (e.g., genes, proteins, hormones, among other molecules). In Boolean networks, variables can only take one of two possible values, 0 or 1, and their dynamics is described by

$$x_i(t+1) = f_i(x_1(t), \ldots, x_n(t)), \quad (1)$$

where $x_i(t+1)$ represents the value of variable i at the time $t+1$ as a Boolean function f_i of its n regulators $x_i(t)$,..., $x_n(t)$ at the current time. In particular, we use synchronous Boolean networks, where the value of all variables is updated at each time step.

The set of all variables time t is a network state. The number of network states in any network is equal to 2^v, where v is the number of variables. Stationary network states are known as attractors. Single-state, stationary configurations are known as fixed-point attractors whereas a set of network states that orderly repeat correspond to cyclic attractors. The size of an attractor is equal to the number of network states that conform such attractor.

For this work it is important to note that not all interactions in a network structure are necessarily functional. An interaction from a variable i to a variable j is considered functional, if j can change its value due to a change only in the value of i. The interaction sign is positive, if the change in the value of j goes in the same direction as the change in the value of i, and is negative otherwise (Fig. 1A; see Supplementary Methods for formal definitions). Note that a variable can act as a positive regulator and as a negative regulator of the same variable in different network states. According to some authors, interactions where a variable is a positive and negative regulator of another variable are not common in real molecular networks[21,28–30]. On the other hand, non-functional interactions, where a regulator does not influence the value of the regulated variable, do not provide any meaningful

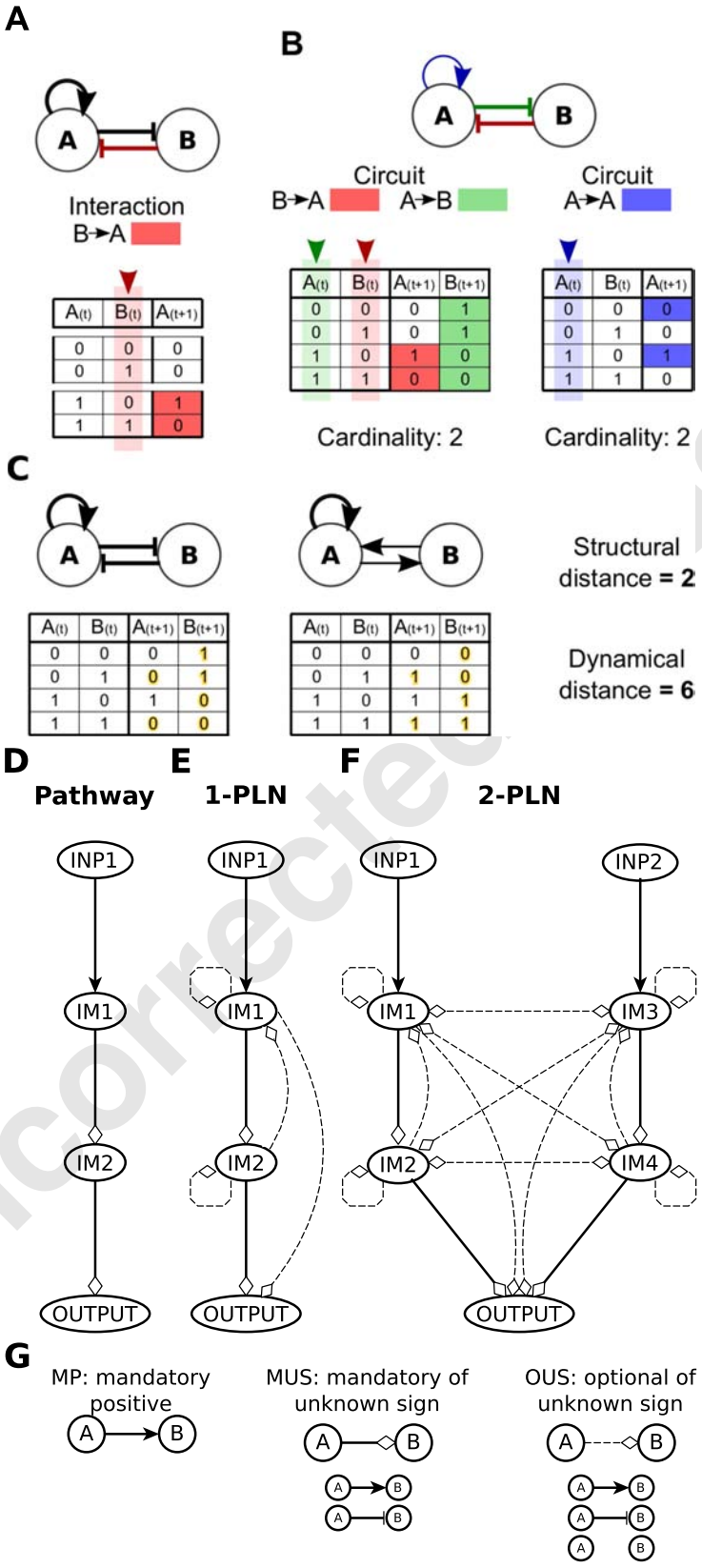


Figure 1. Basic concepts and Pathway-like networks. (**A**) Analysis of the functionality of the interaction from B to A (red link in the interaction graph above). Notice that in the last pair of lines of the table, $A(t+1)$ and $B(t)$ values are opposite, while $A(t)$ is equal to 1 in both lines, indicating that a change, only in the value of B (highlighted in light red) can change the value of A in the next time step (highlighted in red). Thus, the interaction is functional and negative. (**B**) Above, the interaction graph of a network containing two positive feedback circuits, one between A and B (red and green interactions) and the other of A with itself (blue

interaction). As observed below, both feedback circuits are functional, as there exists at least one network state (the network states are conformed by all the combination of $A(t)$ and $B(t)$ values in the right side of the tables) where all the interactions of each circuit are functional. The cardinality is equal to the sum of the network states where the feedback circuit is functional. Thus, the combination of functionalities of this network comprises a positive feedback circuit from *A* to *A* with a cardinality of two, and a positive feedback circuit between *A* and *B* with a cardinality of two. (**C**) Dynamical and structural distance between two networks. Above, the interaction graphs of two different networks. The structural distance is equal to the differences between the interaction graphs of the networks. Below, the state diagrams of the networks. The dynamical distance is equal to the sum of the differences (highlighted in yellow) between the state diagrams of both networks. (**D**) Pathway, (**E**) 1-PLN and (**F**) 2-PLN structure. IN*P* = INPUT and IM = Intermediary Molecules. (**G**) Interaction types considered in this work to construct pathways, 1-PLNs and 2-PLNs: mandatory positive (MP), mandatory of unknown sign (MUS) and optional of unknown sign (OUS). Mandatory interactions are always present, while optional interactions can be either present or not. Positive interactions are always positive. Unknown sign interactions can be either positive or negative.

information. Hence, in the networks analyzed here, we forbid both non-functional interactions and interactions where a regulatory variable has both a positive and a negative influence over another variable.

A feedback circuit is a set of directed interactions forming a closed path. Feedback circuits can be positive or negative. The sign of a feedback circuit is given by the signs of its interactions. A circuit is positive if it has an even number of negative interactions, it is negative otherwise. The sole presence of a circuit in a network does not guarantee its functionality. Thus, a circuit is considered functional if all interactions of the circuit are functional in a set of common network states[25]. Notice that using our sign and functionality definitions, there are four categories of feedback circuits, positive functional, negative functional, positive non-functional and negative non-functional. Furthermore, in this work, functional feedback circuits are characterized by the sign and number of network states where the circuit is functional (cardinality). The combination of the functionalities of feedback circuits is the set of all circuits functionalities present in a given network (Fig. 1B).

Here, we compare the structural and dynamical distance between networks. The structural distance is the sum of connectivity differences between the interaction graphs of two different networks. In particular, we considered as a connectivity difference any difference in the number or the sign of the interactions of two interaction graphs (Fig. 1C). The dynamic of a model can be visualized as a state diagram. A state diagram is a directed graph where each state in the state space represent a network state and is connected to the update state reached after applying to it the Boolean functions associated to the variables. The dynamic distance is a measure of the difference between the state diagrams of two networks. To calculate the dynamic distance we sum the differences in the states reached after applying to each network state the Boolean functions of each network (Fig. 1C).

Pathway-like networks construction. We analyze two types of networks, namely pathways and pathway-like networks (PLNs), both containing different types of interactions identified with the acronyms MUS, OUS and MP, which stand for mandatory unknown sign, optional unknown sign, and mandatory positive, respectively (Fig. 1G). MUS and MP interactions form unidirectional and hierarchical pathway structures (Fig. 1D). PLNs contain MUS, MP and OUS interactions (Fig. 1E,F). We construct two PLNs variants: single (1-PLNs) and double (2-PLNs). 1-PLNs is the set of networks that contains a pathway within its structure and at least one OUS interaction (Fig. 1E). 2-PLNs is the set of networks composed by two parallel pathways regulating the same output and at least one OUS interaction (Fig. 1F). 2-PLNs may have cross-regulation among their constituent pathways, which is a common biological situation.

We used two different approximations to analyze the size and the number of attractors. (1) We simulated each PLN starting from all network states until finding the attractors. (2) We use symbolic algorithms to search for PLNs with a specific number and size of attractors. It is important to note that in most cases, a given network structure can be described by more than one set of Boolean functions. Thus, there is a vast number possible PLNs structural and dynamical variants. Without considering the sign of the interactions, the num$_r$ber of possible structures for a network with v variables is equal to 2^{v^2}. The Boolean functions associated with a variable is 2^{2^r}. where *r* is the number of regulators of the variable. Therefore, the total number of possible Boolean functions for a completely interconnected network with v variables is $2^{v\times 2^r}$. In particular, there are $\approx 4.15 \times 10^{34}$ 2-PLNs. The analysis of such a number of variants is humanly and computationally unfeasible. Thus, we use random sampling or constrained the maximum number of interactions for 2-PLNs analyses (see Supplementary Methods for more details).

Results

Non-hierarchical and non-unidirectional interactions greatly increases the dynamical diversity of pathways. Conventionally, molecular regulation is represented as unidirectional and hierarchical pathways, comprised by an input, internal molecules, and an output (Fig. 1D). To study pathways with more realistic structures, we consider the possibility that the internal molecules may regulate any component inside the pathway (excluding the input). We call this structure a Pathway-like network (PLN). We analyzed single (1-PLNs) and double (2-PLNs) PLNs (Fig. 1E,F). PLNs have non-unidirectional interactions, and the internal molecules are non-hierarchically organized. For simplicity, we refer to PLNs as non-unidirectional and non-hierarchical structures. Regarding their dynamics, PLNs are constructed in such a way that the generation of Boolean functions

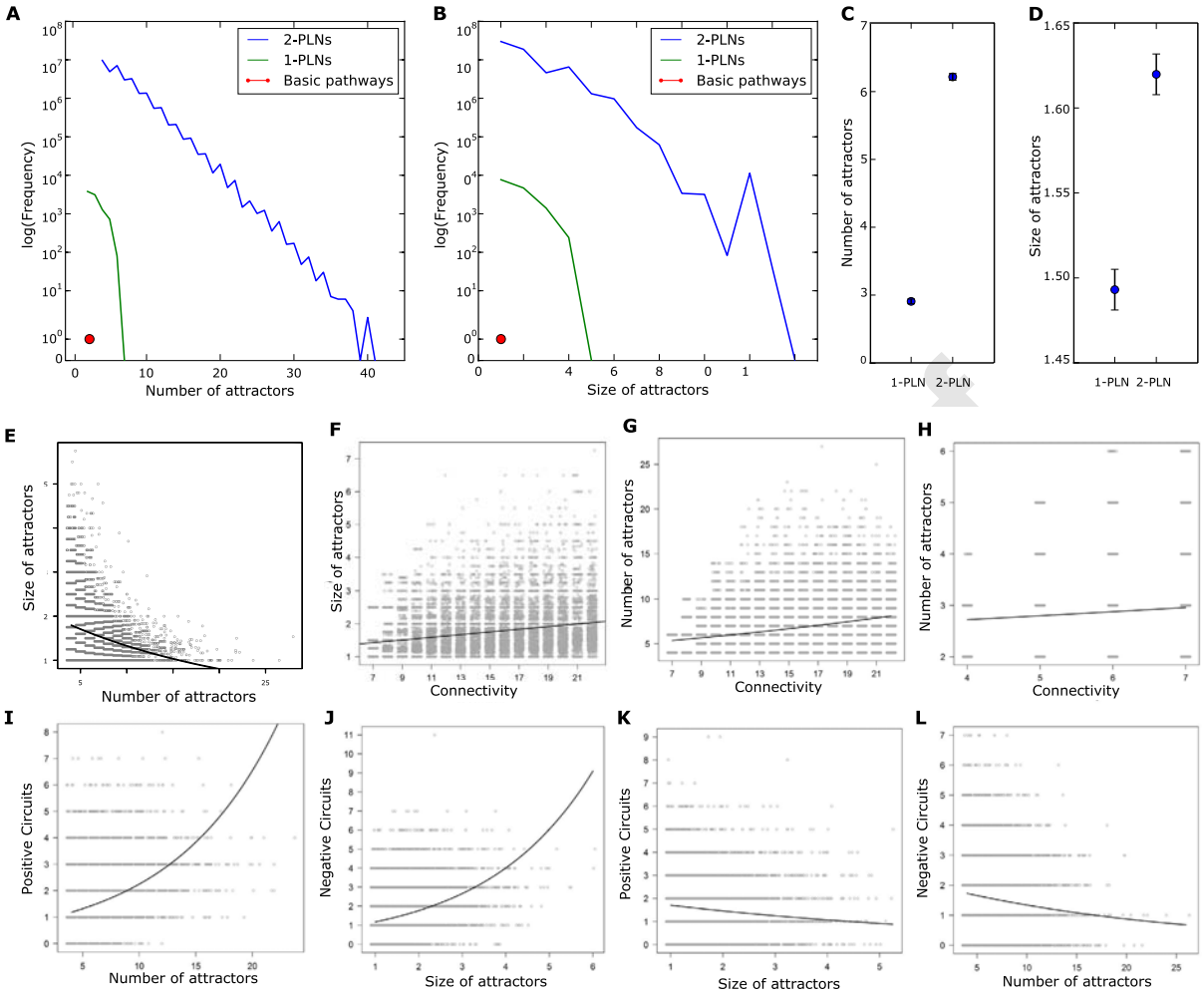


Figure 2. PLNs properties and relations. Distribution of the (**A**) number and (**B**) size of the attractors in pathways, 1-PLNs and 2-PLNs. Mean and confidence interval of the (**C**) mean number and (**D**) mean size of attractors for 1-PLN and 2-PLNs. (**E**) Relation between the number and mean size of attractors of 2-PLNs. (**F**) Mean size of attractors vs. 2-PLNs connectivity. Number of attractors vs. connectivity of 2-PLNs (**G**) and 1-PLNs (**H**). 2-PLNs mean size of attractors vs. quantity of (**I**) positive and (**J**) negative feedback circuits, respectively. 2-PLNs number of attractors vs. quantity of (**K**) positive and (**L**) negative feedback circuits, respectively. In E-L each point represents a single 2-PLN or 1-PLN data, while the line represents the values predicted by Poisson GLM. Points in are displaced the X axis only for visual purpose. Similar results were found in 1-PLNs (Fig. S1).

producing "meaningless" behaviors is forbidden[21,28]. Altogether, PLNs contain realistic structural and dynamical properties (see Methods).

We analyzed the dynamical diversity, measured as the number and the size of attractors in pathways, 1-PLNs and 2-PLNs. The simulation of all pathways, all 1-PLNs and more than 30 millions 2-PLNs randomly selected, showed that the dynamical diversity vastly increases from pathways to 1-PLNs to 2-PLNs (Fig. 2A,B). Because the inputs follow the identity function, the minimum number of attractors is equal to 2linputsl, where inputs is the set of inputs. On the other hand, the maximum number of attractors founded in our simulations are 2, 6, and 40 in pathways, 1-PLNs and 2-PLNs, respectively. This increase is also observed in the mean values, where the mean value of the number of attractors of 2-PLNs is significantly larger than in 1-PLNs ($P < 0.001$; Fig. 2C; see Supplementary information file for detailed information about all statistical results). Similar results can be observed for the size of attractors. Specifically, the maximum sizes are 1, 4, and 13 for pathways, 1-PLNs and 2-PLNs, respectively. Here, too, the mean value of the size of the 2-PLNs attractors is significantly larger ($P < 0.001$; Fig. 2D). Note that both the mean size and mean number of attractors are much closer to their minimum value than to their maximum value. This indicates that most networks have a small number of attractors of small size; and at first sight it seems that they might fit a long-tailed distribution. However, these data do not fit power-law, logarithmic, exponential, normal or Poisson distributions. Nevertheless, our results clearly show that the overall diversity of dynamical behaviors grows from pathways to 1-PLNs to 2-PLNs, due to the addition of non-unidirectional and non-hierarchical interactions.

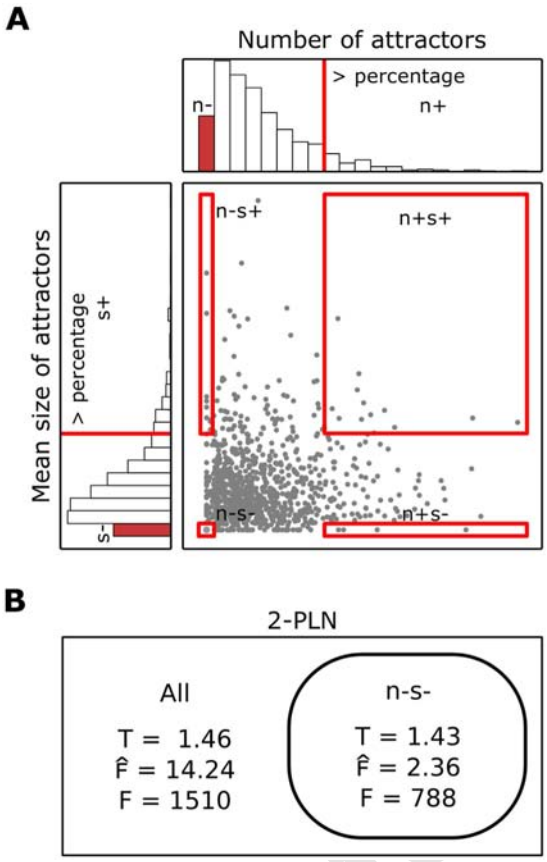


Figure 3. PLNs attractors properties regulation by feedback circuits. (**A**) Classification of PLNs according to the number and mean size of the attractors: *n+* more attractors than 70%, 80%, 90%, 95% or 99% of the PLNs, *n−* minimum number of attractors, *s+* mean size of attractors bigger than 70%, 80%, 90%, 95% or 99% of the PLNs and *s−* fixed-point attractors. (**B**) Total number of combinations of functionalities in 50 random sampling of 2,000 2-PLNs and 2,000 n−s− 2-PLNs (**F**). Mean number of combinations of functionalities contained in 2-PLNs structures and in n−s− 2-PLNs structures obtained from a sampling of one million 2-PLNs randomly generated ($\hat{F}$). Mean number of 2-PLNs and n−s− 2-PLNs structures that contained each combination of functionalies obtained from a sampling of one million 2-PLNs randomly generated (T).

Feedback circuits' role in the regulation of the PLNs attractors properties. We noticed that there is a statistically significant negative relation between the mean size and the number of attractors ($P < 0.001$; Figs 2E and S1A), suggesting that both the size and the number of attractors are regulated by the same property, but in opposite directions. Then, we noticed that as we increase the number of non-unidirectional and non-hierarchical interactions, the size of the attractors increases in 2-PLNs ($P < 0.001$; Fig. 2F) and the number of attractors increases in both 1-PLNs and 2-PLNs ($P < 0.001$; Fig. 2G,H), showing that the property regulating the size and number of attractors, increases as we increase the connectivity of the PLNs. Positive feedback circuits are necessary for multistability, while negative feedback circuits are necessary to produce cyclic attractors[12,13] and adding more non-unidirectional and non-hierarchical interactions create more feedback circuit. Thus, it seems reasonable to think that the appearance of feedback circuits in PLNs structures is responsible for the increase in the dynamical diversity, and that depending on their sign, the feedback circuits will increase the size or the number of attractors. Indeed, positive feedback circuits are positively related with the number of attractors ($P < 0.001$), and negative feedback circuits are positively related with the size of attractors ($P < 0.001$;; Fig. 2I,J). Furthermore, positive feedback circuits have a negative relation with the size of attractors ($P < 0.001$), while negative feedback circuits have a negative relation with the number of attractors ($P < 0.001$; Figs 2K,L and S1B–E), explaining the negative relation between number and size.

To better analyze how feedback circuits regulate the size and number of attractors according to their number and size of attractors in four categories. (1) PLNs with the minimum number of attractors, whose mean size is bigger than the mean size of certain percentage of the total set of PLNs analyzed (n−s+). (2) PLNs with more attractors than certain percentage of the PLNs, all fixed-point attractors (n+s−). (3) PLNs with the minimum number attractors, all fixed-point (n−s−). (4) PLNs with more attractors than a percentage of the PLNs, whose size is bigger than the same percentage of the PLNs (n+s+) (Fig. 3A). We classified all 1-PLNs and selected 10,000 2-PLNs of each category from a sample of over six million 2-PLNs that were randomly generated. We used five percentages for the size and the number of attractors, namely 70%, 80%, 90%, 95% and 99%. In 1-PLNs is not

	2-PLNs			
	Total ± CI	Ratio ± CI	Positive ± CI	Negative ± CI
70%				
n−s−	3.34 ± 0.04	1.25 ± 0.02	1.71 ± 0.02	1.62 ± 0.03
n−s+	3.64 ± 0.04	0.74 ± 0.02	0.98 ± 0.02	2.64 ± 0.04
n+s−	3.77 ± 0.04	2.53 ± 0.02	3.13 ± 0.04	0.65 ± 0.02
n+s+	4.72 ± 0.04	1.11 ± 0.02	2.25 ± 0.03	2.45 ± 0.03
80%				
n−s−	3.34 ± 0.04	1.25 ± 0.02	1.71 ± 0.02	1.62 ± 0.03
n−s+	3.70 ± 0.04	0.67 ± 0.02	0.99 ± 0.02	2.70 ± 0.03
n+s−	4.46 ± 0.04	2.71 ± 0.02	3.69 ± 0.04	0.72 ± 0.02
n+s+	5.66 ± 0.05	1.16 ± 0.02	2.87 ± 0.03	2.76 ± 0.03
90%				
n−s−	3.34 ± 0.04	1.25 ± 0.02	1.71 ± 0.02	1.62 ± 0.03
n−s+	3.74 ± 0.04	0.66 ± 0.02	1.02 ± 0.02	2.72 ± 0.03
n+s−	4.71 ± 0.04	2.83 ± 0.02	3.88 ± 0.04	0.71 ± 0.02
n+s+	NA	NA	NA	NA
95%				
n−s−	3.34 ± 0.04	1.25 ± 0.02	1.71 ± 0.02	1.62 ± 0.03
n−s+	4.65 ± 0.04	0.62 ± 0.02	1.31 ± 0.02	3.39 ± 0.04
n+s−	5.08 ± 0.04	3.00 ± 0.02	4.28 ± 0.04	0.73 ± 0.02
n+s+	NA	NA	NA	NA
99%				
n−s−	3.34 ± 0.04	1.25 ± 0.02	1.71 ± 0.02	1.62 ± 0.03
n−s+	5.17 ± 0.05	0.62 ± 0.02	1.51 ± 0.02	3.66 ± 0.04
n+s−	5.51 ± 0.05	3.30 ± 0.02	4.68 ± 0.04	0.76 ± 0.02
n+s+	NA	NA	NA	NA

Table 1. 2-PLNs feedback circuits. Positive/negative ratio and number of positive and negative feedback circuits in 2-PLNs divided by category (n+s+, n−s+, n+s− and n−s−) using different thresholds for the number and the mean size of attractors. The number of positive and negative feedback circuits within the same category, have significant differences in all cases ($P < 0.001$). Differences of the total and the ratio between categories is shown in the Supplementary Material. NA stand for not enough data available. The "Total" column corresponds to the sum of positive and negative feedback circuits.

	1-PLNs			
	Total ± CI	Ratio ± CI	Positive ± CI	Negative ± CI
80%				
n−s−	2.59 ± 0.12	1.06 ± 0.03	1.24 ± 0.08	1.35 ± 0.08
n−s+	2.57 ± 0.07	0.57 ± 0.03	0.29 ± 0.02	2.29 ± 0.07
n+s−	2.74 ± 0.09	2 ± 0	2.46 ± 0.09	0.29 ± 0.03
n+s+	NA	NA	NA	NA
90%				
n−s−	2.59 ± 0.12	1.06 ± 0.03	1.24 ± 0.08	1.35 ± 0.08
n−s+	2.52 ± 0.10	0.75 ± 0.07	0.15 ± 0.02	2.37 ± 0.09
n+s−	2.88 ± 0.13	2 ± 0	2.63 ± 0.12	0.25 ± 0.04
n+s+	NA	NA	NA	NA
95%				
n−s−	2.59 ± 0.12	1.06 ± 0.03	1.24 ± 0.08	1.35 ± 0.08
n−s+	2.67 ± 0.17	NA	0	2.67 ± 0.17
n+s−	3.00 ± 0.00	NA	3.00 ± 0.00	0
n+s+	NA	NA	NA	NA

Table 2. 1-PLNs feedback circuits. Positive/negative ratio and number of positive and negative feedback circuits in 1-PLNs divided by category (n+s+, n−s+, n+s− and n−s−) using different thresholds for the number and the mean size of attractors. The number of positive and negative feedback circuits within the same category, have significant differences in all cases ($P < 0.001$ except n−s− for which $P < 0.057$). Differences of the total and the ratio between categories is shown in the Supplementary Material. It was not possible to do statistical analyses with 95% due to the lack of variability. The "Total" column corresponds to the sum of positive and negative feedback circuits. NA stand for not enough data available.

possible to define 99% and 70%, due to the limited amount of data, and we did not find n+s+ 1-PLNs for any percentage and for n+s+ 2-PLNs using 90% or higher. Then, to see if the quantity of feedback circuits in a PLN is important to determine the number and the size of the attractors, we compared the total number of feedback circuits of n+s+, n+s−, n−s+ and n−s−. In 2-PLNs, the total number of feedback circuits consistently and significantly increase from n−s− to n−s+ to n+s− to n+s+ (Tables 1 and S1), suggesting that as the number of feedback circuits increases, the number and size of attractors tends to increase too. According to Kwon *et al.*[31], the positive/negative feedback circuits ratio provides the trend of the number and the size of attractors, with larger ratio values for networks with more attractors of smaller size. If this is the case, n+s− PLNs should have a bigger positive/negative feedback circuits ratio than n−s+. On the other hand, n−s− and n+s+ could have a balance between positive and negative feedback circuits. As observed in Tables 1, 2, S1 and S2), this is indeed the case in both 1-PLNs and 2-PLNs using all percentages ($P < 0.001$). From the circuits ratio result, we can expect the PLNs n+s−to have more positive feedback circuits and fewer negative feedback circuits, the opposite behavior for n−s+, and a similar number of positive and negative feedback circuits in the n+s+ and n−s−. As observed, this is the case in both 1-PLNs or 2-PLNs (Tables 1 and 2). Thus, our results support the idea that the positive/negative feedback circuits ratio provides the correct trend for the size and the number of attractors. Thus, according to our results, the feedback circuits ratio do provide the correct trend of the number and size of the attractors in a network.

The combination of the functionalities of the feedback circuits regulates the attractors size and number. It is important to note that because the feedback circuits ratio provides a statistical relation of how the number and size of attractors will behave, there could be some instances where the size and number are not consistent with the feedback circuits ratio. In fact, we detected PLNs that behaved opposite to what was expected from their feedback circuits ratio. For example, we found PLNs n+s− with a lower positive/negative feedback circuits ratio than the mean ratio in PLNs n−s+ and vice versa (e.g., Supplementary Fig. S2). Observe that the mere presence of a feedback circuit in the structure does not guarantee the expected behavior unless the circuit is functional[8,25]. Then, the feedback circuits ratio might not predict the correct trend when some of the circuits are not functional. For a circuit to be functional, all interactions within such a circuit should be functional in a shared set of network states[25] (Fig. 1B). To study whether the functionality of the circuits is behind the unexpected behaviors, we analyzed the functionality of feedback circuits of all 1-PLNs and a sampling of one million 2-PLNs. Indeed, we found that not all feedback circuits in the PLNs are functional. However, considering only functional feedback circuits do not eliminated all the unexpected behaviors (e.g., Supplementary Fig. S2). This result indicates that the circuits ratio and functionality is not enough to understand how the size and the number of attractors are regulated. Thus, we decided to look for complementary properties that could allow us to better understand how the number and size of attractors are regulated.

We noticed that the effect the multiple feedback circuits have over the number and size of the attractors depends, not only on their sign and functionality, but also on how circuits couple to each other. In particular, we observed that the same feedback circuits in the same network structure can be functional in a different number of network states (i.e., a circuit can have different cardinalities) and changes in the circuits cardinality can (1) modify the number and the size of the attractors and (2) modify the cardinality of other circuits (Fig. S3). Thus, the cardinality of the circuits could be important to understand how multiple feedback circuits couple and determine the number and size of attractors. Accordingly, we defined the combinations of the functionalities of feedback circuits (hereinafter named combinations of functionalities) of a PLNs as the set of all its circuits functionalities, characterized by sign and cardinality (Fig. 1B). If a specific number and size of attractors is produced only by certain combinations of functionalities, a set of PLNs with the same number and size of attractors, such as n−s− PLNs, should have a reduced number of combinations of functionalities than a set of PLNs with different number and size of attractors. Thus, to study if only certain combinations of functionalities can produce a specific number and size of attractors, we compared the number of combination of functionalities in (1) all 1-PLNs against all n−s− 1-PLNs and in (2) 50 samples of 2,000 2-PLNs against 2,000 n−s− 2-PLN, randomly selected from 50 samples of 100,000 2-PLNs randomly generated. Indeed, there are only 8 combinations of functionalities in n−s− 1-PLNs and 62 in 1-PLNs. Similarly, there are significantly fewer combinations of functionalities in n−s− 2-PLNs (788 ± 7.74) than in 2-PLNs ($1{,}510 \pm 11.07$; $P < 0.001$; Fig. 3B). This result supports the idea that specific number and size of attractors are produced by specific combinations of functionalities. Then, if only certain combinations of functionalities are capable to produce a specific number and size of attractors, only some of the combinations of functionalities contained in a structure should produce attractors of a specific size and number. We found that by selecting the n−s− PLNs, the mean number of combinations of functionalities contained in each structure in all 1-PLNs and in one million 2-PLNs randomly selected diminished from 8.85 ± 21.38 to 2.66 ± 3.65 and from 14.24 ± 0.199 to 2.36 ± 0.146 in 1-PLNs and 2-PLNs, respectively (Fig. 3B). This reduction is significant in the 2-PLNs case ($P < 0.001$), demonstrating that over the large number of existing combinations of functionalities in a structure, only some of them are able to produce a specific number and size of attractors.

Observe that networks with different structures can produce the same combination of functionalities (Fig. S3A,B). If the combination of functionalities regulate the attractors size and number, different PLN structures producing the same combination of functionalities should also be able to produce the same number and size of attractors. The latter would be better supported if each combination of functionalities is, on average, contained in the same number of structures in a set of PLNs with the same size and number of attractors and in a set of PLNs with different size and number of attractors. To analyze if this is the case, we compared the mean number structures containing each combination of functionalities in (1) all 1-PLNs structures against all n−s− 1-PLN structures and in (2) the structures 2-PLNs against the structures of n−s− 2-PLN, from a sample of one million 2-PLNs randomly selected. Each combination of functionalities is contained in exactly two structures in both n−s− 1-PLNs and the complete set of 1-PLNs. Similarly, the combinations of functionalities of 2-PLNs and

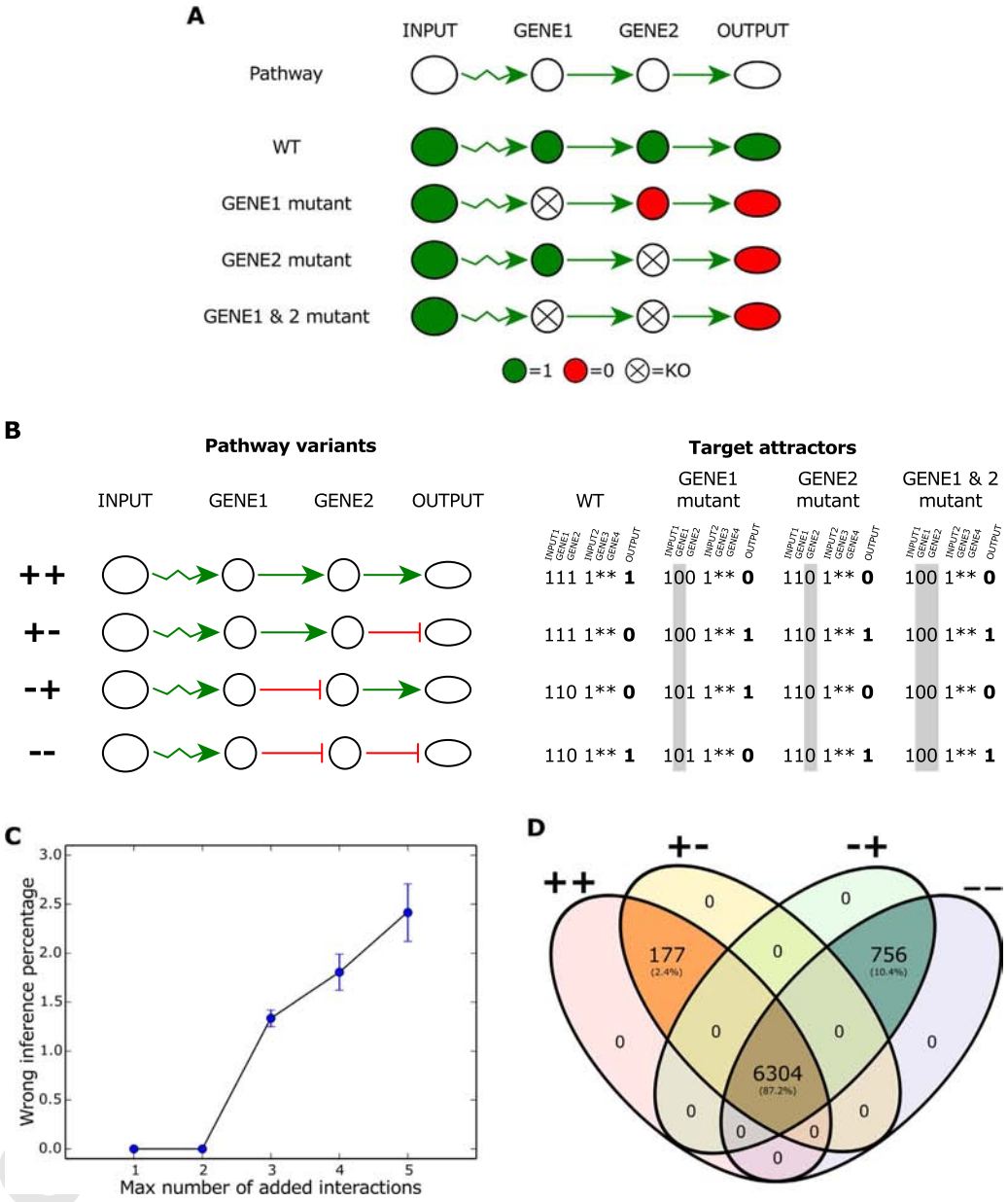


Figure 4. Characterization of the PLNs that produce the epistasis results. (**A**) Boolean interpretation of the epistasis analysis using the ++ pathway variant shown in (**B**). Target attractors for each of the four pathway variants using the epistasis analysis. Mutated genes are highlighted in grey. For 1-PLNs we only consider INP1, GENE1/2 and OUTPUT values. Epistasis analysis do not consider the possibility of a second parallel and cross-talking pathway. Thus, the asterisks represent unknown values for GENE3 and GENE4 in the 2-PLNs case. Input values are considered equal to 1 because the epistasis analysis assumes that the input values are constant during the experiment[1]. (**C**) Percentage of 2-PLNs that produce the target attractors of different pathway variant than the pathway variant contained in the 2-PLN as we added more interactions the unidirectional and hierarchical pathway structure. (**D**) Percentage of combinations of functionalities shared between the PLNs that produced each of the four sets of target attractors.

n−s− 2-PLNs are contained in 1.46 ± 0.005 and in 1.43 ± 0.0022 structures (Fig. 3B), respectively, showing no significant differences ($P < 0.331$). By doing an analysis of individual PLNs, we find some instances, in which the same combination of functionality produce a different number or size of attractors. We looked for, but did not find, other complementary properties, such as additional interactions, that allows to completely understand how the number and the size of the attractors is regulated. However, our results strongly suggest that network structures with the same number of variables and with the same combination of functionalities, in general, can produce the same number and size of attractors.

Combinations of the functionalities of feedback circuits and the analysis of epistasis. It is important to note that our definition of combination of functionalities is independent of the specific network states that are attractors. Thus, a complementary way to test if the combinations of functionalities regulate the number and the size of attractors, is to search for networks with the same number of attractors of the same size, but whose attractors are represented by different network states. These networks should be produced by the same or similar sets of combinations of functionalities.

We noticed, that epistasis analysis, as described by refs 1,18,19, provide this possibility. Briefly, epistasis is a term used when the phenotype of an allele is masked by an allele in another locus. The gene with the allele whose phenotype persists when the alleles of both loci are present is called epistatic gene, while the other is the hypostatic gene. Epistasis analysis uses a simple set of two rules to order the epistatic and hypostatic genes[1,18,19]. First, in a double-mutant experiment, the epistatic gene is upstream and positively regulates the downstream gene when the two genes used in the double-mutant display a characteristic single-mutant phenotype under the same condition. And second, in a double mutant experiment, the epistatic gene is downstream and is negatively regulated by the upstream gene when the two genes display a characteristic single mutant phenotype under different conditions. The epistasis analysis can be formalised in Boolean terms in a straightforward way (Fig. 4A). Observe that the possible combinations of positive and negative interactions in a pathway already gives four pathway variants that have the same number and size of attractors, but the attractors of each pathway variant correspond to different network states. Previous works had characterized the expected results of the four pathway variant[1,18,19]. Thus, we used the expected results of each pathway variant by the epistasis analysis to further analyze the importance of the combinations of functionalities in the regulation of the size and number of attractors.We named each pathway variant according to the sign of the regulation from GENE1 to GENE2 and from GENE2 to the OUTPUT, as $++$, $+-$, $-+$ and $--$. Then, we interpreted the epistasis analysis expected results for each pathway variant as the set of target attractors in our PLNs (Fig. 4B) and analyzed the number of 1-PLNs and 2-PLNs that achieved each of the four possible sets of target attractors.

The number of PLNs able to produce the target attractors in the 1-PLNs is 60 for the $++$ and $+-$ variants and 68 for the $-+$ and $--$ variants. We were unable to perform an exhaustive 2-PLNs search because of the astronomical number of redundant 2-PLNs found. Restricting our search to 2-PLNs with a maximum of five extra interactions compared to the pathway structure, we found more than 4.731×10^7 2-PLNs that produced each set of target attractors. In the 1-PLNs case, the target attractors of a pathway variant are only obtained when the same pathway variant is contained in the PLN. On the other hand, $\approx$2.41% of the 2-PLNs produced wrong inferences. We considered PLNs producing the target attractors of a pathway variant different from the pathway variant contained in its structure as wrong inferences. This demonstrates that epistasis analysis can produce wrong regulatory inferences when analyzing non-unidirectional and non-hierarchical networks, as stated before by our group[21]. The wrong inferences increase as we added more interactions (Fig. 4C) and are produced thanks to the added interactions between the parallel pathways contained in the 2-PLNs. It is interesting to note, that, apparently, when there are wrong inferences, the extra interactions produce an alternative pathway that conformed with the expected pathway variant but that contained some intermediary steps between GENE1/2 or between GENE2 and OUTPUT (see some examples in Fig. S4). However, the alternative pathway by itself is not sufficient to produce the expected results as the alternative pathway can be created by adding one interaction and inconsistencies between the target attractors and the pathway variant contained in the PLNs appear only when we added three or more interactions (Fig. 4C). Thus, our results demonstrate that epistasis analysis produce wrong and incomplete inferences in non-hierarchical and non-unidirectional networks, but when a network has a low connectivity, wrong regulatory inferences are scarce.

The combinations of functionalities that produced the four target attractors are almost the same in both 1-PLNs and 2-PLNs. In 1-PLNs all four target attractors share one combination of functionalities (see Fig. S5). This is a meaningful result as that is the only combination of functionalities that produced the target attractors of the variants $++$ and $+-$ and one of the two combination of functionalities that produce the target attractors of the variants $-+$ and $--$. In the 2-PLNs case, there are 6,481 combinations of functionalities that produced the target attractors of the variants $++$ and $+-$ and 7,060 that produced the target attractors of the variants $-+$ and $--$. 6,304 combinations functionalities are shared by the four pathway variant (Fig. 4D). This is an astonishing result that greatly supports the importance of the combinations of functionalities to determine the attractors properties, as the target attractors vary for each pathway variant, indicating that the common feature among the PLNs found are the number and the size of attractors.

Comprehensive characterization of PLNs. Having multiple networks producing the same set of results raises a problem for the analyses of molecular regulation by methods, such as the epistasis analysis, as they cannot distinguish between these networks. Hence, we did an exploration of which properties could be useful to distinguish between these networks. For this characterization, we used all 1-PLNs and 2-PLNs with no more than 2 interactions added to the unidirectional and hierarchical pathway structure, because comparison of 2-PLNs with three or more extra interactions was extremely challenging or computationally impossible.

First, we analyzed if structurally similar PLNs followed similar dynamics (Fig. 1C). As observed in Fig. 5A, dynamical and structural distances are weakly related. Because the relation is weak, structurally close PLNs can have large dynamical distances and vice versa. Thus, even when dynamical and structural properties are related, this relation is not sufficient to distinguish between different network strucutres[15,32–34]. Then, we noticed that PLNs producing the expected results of the same pathway variant are dynamically closer than networks producing the results of a different pathway variant. Likewise, PLNs with the same combination of functionalities are contained in clusters of PLNs with the same structure. Interestingly, PLNs within the same cluster and with the same combination of functionalities are among the more similar networks at the dynamic level (Fig. 5B). Thus,

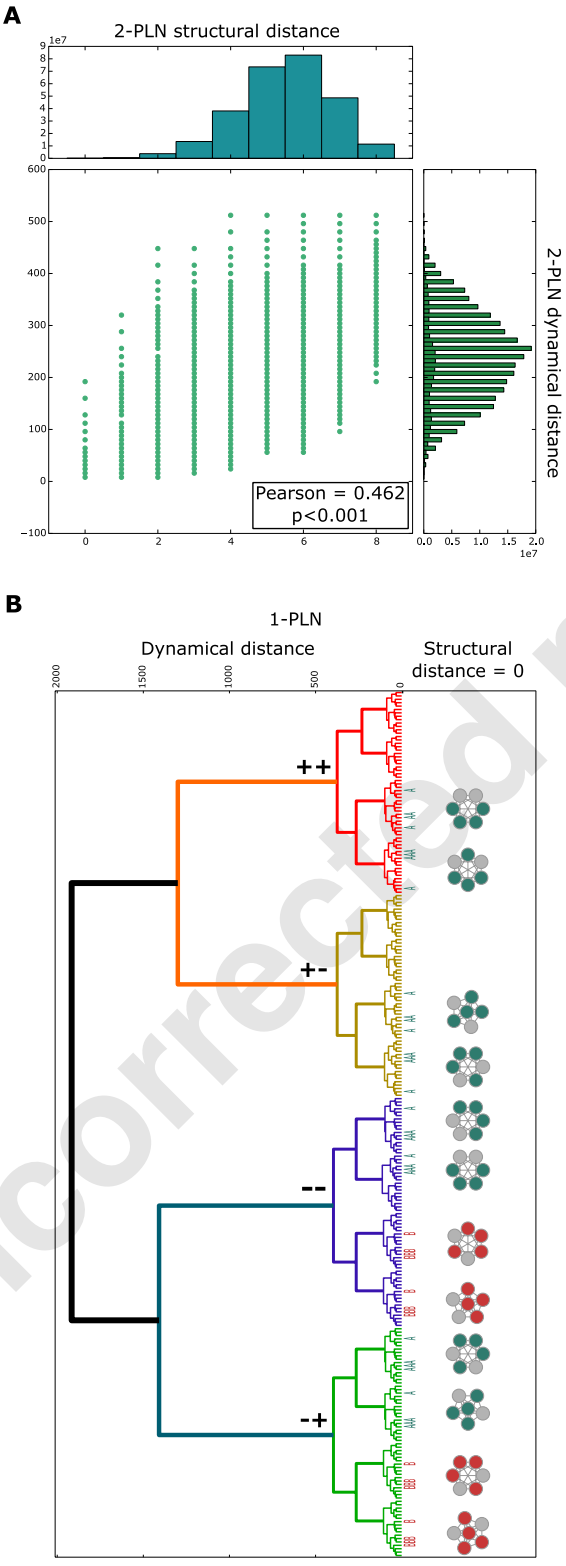


Figure 5. PLNs characterization. (**A**) 2-PLNs relationship between dynamical and structural distance. (**B**) On the left side, 1-PLNs dendogram using the dynamical distance. Each leaf of the dendogram represent a 1-PLN. As observed, 1-PLNs containing the results of the same pathway variant form distinguishable clades, colored with red (++ variant), yellow (+− variant), purple (−− variant) and green (−+ variant). The blue A and red B beside the dendogram represent the two combinations of functionalities found in 1-PLNs and are aligned with the 1-PLNs that contain them. 1-PLNs not aligned with an A or a B do not have any functional circuit. On the right side, clusters formed of 1-PLNs with a structural distance of 0. The blue and red nodes of the clusters are approximately aligned with their corresponding 1-PLNs marked with the blue As and red Bs in the dendogram. Grey nodes of the clusters correspond to PLNs with no functional circuits.

PLNs with the same combination of functionalities and the same structure form small groups with extremely similar dynamic properties. Based on these results, we believe that studying the characteristics of these regions could be an important step towards a better and more general understanding of molecular regulation and could allow for a better characterization of regulatory networks to improve the scope of traditional methods for the inference of regulatory interactions. The analysis of such regions and their integration in epistasis analysis context remain as interesting questions for future research.

Conclusions and Discussion

We characterized the effect of adding certain interactions within unidirectional and hierarchical pathway structures. These extra interactions create realistic regulatory structures with a resulting non-unidirectional and non-hierarchical organization containing motifs that we named Pathway-like Networks (PLNs). Additionally, we included certain procedures to ensure the creation of only biologically meaningful dynamics[3,22,28]. As a result, PLNs have realistic structural and dynamical properties. We used Boolean formalism to model the dynamic of these networks for many reasons, such as their simplicity and the fact that they have been successfully used to model many gene regulatory networks[10,11,22–24]. However, Boolean formalism has some limitations that should be addressed in the future, for example, in certain cases the level of expression or concentration of genes and proteins produce regulatory interactions that can not be modeled in Boolean terms (e.g., aggravating and synthetic interactions). Despite the limitation of the Boolean formalism, our work clearly shows that PLNs have a great dynamical diversity, characterized by the number and the size of attractors.

The explosion in the dynamical possibilities of the PLNs was expected from previous works. For example, Kauffman[9] hypothesized that the number of attractors in random Boolean networks increase as $\sqrt{v}$. Later, it was found that the number of attractors in Boolean networks increases faster than any power law as the number of variables increases[35]. A strict comparison between these proposals and our results is difficult, because both Samuelsson and Kauffman's proposals were based on studies with a fixed number of connections, while our networks have a variable connectivity. Nonetheless, our results clearly show that the dynamical diversity of PLNs is huge, compared with the dynamical diversity of unidirectional and hierarchical pathways.

We then focused on the role of network motifs, specially feedback circuits, in the size and the number of attractors. We found that, as the number of positive feedback circuits increases, the number of attractors increases and the size of the attractors decreases. We also observed the exact opposite relation between the number of negative feedback circuits and the number and size of attractors. These results were expected considering that positive and negative feedback circuits are required for multistability and oscillations[12,13]. As it has been reported[31], the positive/negative feedback circuits ratio give the correct trend for the number and the size of the attractors. These results are interesting, but they do not explain how feedback circuits in PLNs, which can have multiple coupled feedback circuits, regulate the size and number of attractor. Thus, we looked for a more mechanistic understanding of the regulation of the size and the number of attractors by analyzing the coupling of feedback circuits. We found that the combination of functionalities (i.e., the way feedback circuits couple) is a key regulator of the number and the size of attractors. In general, PLNs with the same combination of functionalities can produce the same number and size of attractors, independently of its structure.

It is important to note that the same combination of functionalities can produce attractors of different size and number, indicating that the combination of functionalities definition does not capture completely the regulation of the size and the number of attractors, leaving many interesting issues for future research. Some of these issues are, if an extended or modified definition of the combination of functionalities would allow to completely understand how multiple circuits regulate the size and the number of attractors. For example, does considering other properties, such as the functionality of interactions between variables of different circuits could improve our understanding of how circuits regulate the number and the size of attractors? It might also be that the property regulating the size is not the same property that regulates the number of attractors. Another important point to for future research is that we compared networks with the same number of variables, but it could be that the same combination of functionalities in a network with different variables will produce attractors with different properties. Hence, we wonder if there is a way to characterize the number and size of the attractors in a network, independently of the number of variables in such a network. Anyhow, we believe that studying how multiple feedback circuits couple and modify the properties of a network, will provide the answer to many of these questions. Thus, it is worthy of further research and could provide insightful information about molecular regulation in general.

In accordance with previous observations[15,23,36], we found that several PLNs with different structures are capable of producing the same number, size, and even the same set of attractors. This result emphasizes how limited are the traditional methods for the analysis of experimental results, such as the epistasis analysis[1,18,19]. First, because such analyses consider only a restricted number of possible networks, they are not well suited to deal with the huge diversity of possible dynamic behaviors in real molecular networks. Second, because such methods are unable to distinguish between alternative networks producing the same set of attractors. Thus, we searched for the number of PLNs that produced the epistasis results. In 2-PLNs, the number of network that produced the same set attractors was so huge, that we needed to constrain our search to a limited number of PLNs structures. It is important to remember that all these networks produce exactly the same attractors, which represent the results expected by epistasis analysis. As a consequence, we can conclude that with the use ofepistasis analysis multiple regulatory structures are indistinguishable[16]. Even more, we find that in some cases they can even produce wrong gene regulation inferences[21]. These incorrect inferences are due to the appearance of alternative pathways that can produce the expected behaviors. There may not be general rules to infer complex network structures, such as PLNs[15,16]. However, our results suggest that it is fundamental to expand the analyses of regulatory interaction, allowing then to include and consider the presence of regulatory motifs, such as feedback circuits. Considering feedback circuits could be specially relevant, as according to our results, networks sharing the same structure

and the same combination of functionalities produce dynamically similar regions. Consequently, in principle, it should be possible to use information, such as the combination of functionalities, to produce better inferences of regulatory structures. In fact, our preliminary studies suggest that by not assuming a unidirectional and hierarchical structure, already improves the inference of regulatory structures[37]. Thus, we believe that a more general understanding of the combination of functionalities and its relation with networks structure and dynamic will open possible ways to study and analyze molecular regulation of biological processes.

References

1. Huang, L. S. & Sternberg, P. W. Genetic dissection of developmental pathways. *WormBook* **14,** 1–19 (2006).
2. Chen, H., Wang, G., Simha, R., Du, C. & Zeng, C. Boolean Models of Biological Processes Explain Cascade-Like Behavior. *Scientific Reports* **7,** 20067 EP- (2016).
3. Zhang, Y., Ouyang, Q. & Geng, Z. Topological origin of global attractors in gene regulatory networks. *Science China Physics, Mechanics & Astronomy* **58,** 1–8 (2014).
4. Barabasi, A.-L. & Oltvai, Z. N. Network biology: understanding the cell's functional organization. *Nat Rev Genet* **5,** 101–113 (2004).
5. Milo, R. *et al.* Network Motifs: Simple Building Blocks of Complex Networks. *Science* **298,** 824–827 (2002).
6. Barabasi, A.-L. & Albert, R. Emergence of scaling in random networks. *Science* **286,** 509–512 (1999).
7. Thomas, R. & Richelle, J. Positive feedback loops and multistationarity. *Discrete Applied Mathematics* **19,** 381–396 (1988).
8. Thomas, R., Thieffry, D. & Kaufman, M. Dynamical behaviour of biological regulatory networks-I. Biological role of feedback loops and practical use of the concept of the loop-characteristic state. *Bull Math Biol* **57,** 247–276 (1995).
9. Kauffman, S. Metabolic stability and epigenesis in randomly constructed genetic nets. *J Theor Biol* **22,** 437–467 (1969).
10. Espinosa-Soto, C., Padilla-Longoria, P. & Alvarez-Buylla, E. R. A gene regulatory network model for cell-fate determina-tion during arabidopsis thaliana flower development that is robust and recovers experimental gene expression profiles. *The Plant Cell* **16,** 2923–2939 (2004).
11. Albert, R. & Othmer, H. G. The topology of the regulatory interactions predicts the expression pattern of the segment polarity genes in drosophila melanogaster. *Journal of Theoretical Biology* **223,** 1–18 (2003).
12. Snoussi, E. H. Necessary conditions for multistationarity and stable periodicity. *Journal of Biological Systems* **06,** 3–9 (1998).
13. Richard, A. & Comet, J.-P. Necessary conditions for multistationarity in discrete dynamical systems. *Discrete Applied Mathematics* **155,** 2403–2413 (2007).
14. Aracena, J. Maximum number of fixed points in regulatory boolean networks. *Bulletin of Mathematical Biology* **70,** 1398–1409 (2008).
15. Payne, J. L. & Wagner, A. Function does not follow form in gene regulatory circuits. *Scientific Reports* **5,** 13015 EP- (2015).
16. Phenix, H., Perkins, T. & Kasrn, M. Identifiability and inference of pathway motifs by epistasis analysis. *Chaos* **3,** e15 (2013).
17. Nochomovitz, Y. D. & Li, H. Highly designable phenotypes and mutational buffers emerge from a systematic mapping between network topology and dynamic output. *Proc Natl Acad Sci USA* **103,** 4180–4185 (2006).
18. Roth, F. P., Lipshitz, H. D. & Andrews, B. J. Q&A: Epistasis. *Journal of Biology* **8,** 35 (2009).
19. Avery, L. & Wasserman, S. Ordering gene: function the interpretation of epistasis in regulatory hierarchies. *Trends Genet* **8,** 312–316 (1992).
20. Bateson, W. *Mendel's Principles of Heredity* (Cambridge University Press, Cambridge, 1909).
21. Azpeitia, E., Benitez, M., Padilla-Longoria, P., Espinosa-Soto, C. & Alvarez-Buylla, E. R. Dynamic network-based epistasis analysis: boolean examples. *Frontiers in Plant Sciences* **15,** 2, 92 (2010).
22. Azpeitia, E., Weinstein, N., Benitez, M., Mendoza, L. & Alvarez-Buylla, E. R. Finding Missing Interactions of the Arabidopsis thaliana Root Stem Cell Niche Gene Regulatory Network. *Frontiers in Plant Sciences* **4,** 110 (2013).
23. Benitez, M. & Alvarez-Buylla, E. R. Dynamic-module redundancy confers robustness to the gene regulatory network involved in hair patterning of Arabidopsis epidermis. *Biosystems* **102,** 11–15 (2010).
24. Martinez-Antonio, A., Janga, S. C. & Thieffry, D. Functional organisation of Escherichia coli transcriptional regulatory network. *Journal of Molecular Biology* **381,** 238–247 (2008).
25. Naldi, A., Thieffry, D. & Chaouiya, C. Decision Diagrams for the Representation and Analysis of Logical Models of Genetic Networks, vol. 4695 of Computational Methods in Systems Biology. *Lecture Notes in Computer Science*, 233–247 (Springer Berlin Heidelberg, 2007).
26. Comet, J.-P. *et al.* On circuit functionality in boolean networks. *Bulletin of Mathematical Biology* **75,** 906–919 (2013).
27. Remy, E., Ruet, P. & Thieffry, D. Graphic requirements for multistability and attractive cycles in a boolean dynamical framework. *Advances in Applied Mathematics* **41,** 335–350 (2008).
28. Raeymaekers, L. Dynamics of boolean networks controlled by biologically meaningful functions. *Journal of Theoretical Biology* **218,** 331–341 (2002).
29. Marbach, D., Schaffter, T., Mattiussi, C. & Floreano, D. Generating realistic in silico gene networks for performance assessment of reverse engineering methods. *Journal of Computational Biology* **16,** 229–239, http://dx.doi.org/10.1089/cmb.2008.09TT (2009).
30. La Rota, C. *et al.* A data-driven integrative model of sepal primordium polarity in arabidopsis. *The Plant Cell* **23,** 4318–4333 (2011).
31. Kwon, Y.-K. & Cho, K.-H. Boolean Dynamics of Biological Networks with Multiple Coupled Feedback Loops. *Biophysical Journal* **92,** 2975–2981 (2007).
32. Macia, J., Widder, S. & Sole, R. Specialized or flexible feed-forward loop motifs: a question of topology. *BMC Systems Biology* **3,** 1–18, http://dx.doi.org/10.1186/1752-050 9-3-84 (2009).
33. Conrad, E., Mayo, A. E., Ninfa, A. J. & Forger, D. B. Rate constants rather than biochemical mechanism determine behaviour of genetic clocks. *Journal of The Royal Society Interface* **5,** S9–S15, http://rsif.royalsocietypublishing.org/content/5ZSuppl_1/S9. http://rsif.royalsocietypublishing.org/content/5/Suppl_1/S9.full.pdf (2008).
34. Ingram, P. J., Stumpf, M. P. & Stark, J. Network motifs: structure does not determine function. *BMC Genomics* **7,** 1–12, http://dx.doi.org/10.118 6/14 71-2164-7-108 (2006).
35. Samuelsson, B. & Troein, C. Superpolynomial Growth in the Number of Attractors in Kauffman Networks. *Phys. Rev. Lett*. **90,** 098701 (2003).
36. Ciliberti, S., Martin, O. C. & Wagner, A. Robustness can evolve gradually in complex regulatory networks with varying topology. *PLoS Computational Biology* **23** (2007).
37. Rosenblueth, D. A., Munoz, S., Carrillo, M. & Azpeitia, E. Inference of Boolean Networks from Gene Interaction Graphs Using a SAT Solver. *In Algorithms for Computational Biology,* 235–246 (Springer International Publishing, 2014).

Acknowledgements

We thank Gustavo Ramos, Luis Cauich and Jocelyn Champagnon for statistical advice. We thank Dr. Emmanuel Faure, Dr. Mariana Benitez and Dr. Christophe Godin for their comments and discussion. We also gratefully acknowledge support form PASPA-DGAPA-UNAM and Conacyt grants 221341 and 261225. LM acknowledges the sabbatical scholarships from PASPA-DGAPA-UNAM and CONACYT 251420. NW gratefully acknowledges

support from ABACUS, CONACYT grant EDOMEX-2011-C01-165873. MEMS is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México and received fellowship 400554 from CONACYT.

Author Contributions

E.A., L.M. and E.R.A.B. conceived the project. E.A. and L.M. designed the research. E.A., L.M., N.W., S.M. and M.E.M.S. performed the research. E.A., D.G.T., M.E.M.S. and A.N. analyzed the data. E.A., N.W., L.M., S.M., A.N. and D.R. provided and developed software tools. E.A., M.E.M.S. and D.G.T. prepared the figures. E.A. and L.M. wrote the manuscript with inputs from all authors.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Azpeitia, E. *et al.* The combination of the functionalities of feedback circuits is determinant for the attractors' number and size in pathway-like Boolean networks. *Sci. Rep.* **7**, 42023; doi: 10.1038/srep42023 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Capítulo 5

BoolNetPerturb: Robustness and Plasticity in Regulatory Networks

Robustness in Regulatory Networks

A tutorial for BoolNet and BoolNetPerturb

ME Martinez-Sanchez

1 Introduction

This tutorial explains how to use BoolNet and BoolNetPerturb to study the robustness of Boolean regulatory networks and the biological implications of this analysis.

This tutorial supposes that the reader is familiar with the basic concepts of: * **Boolean regulatory networks**. There are a lot of basic introductions to the topic like: Kaplan & Glass, 1995, Azpeitia 2011 and Albert 2014. CoLoMoTo also published a more advanced review CoLoMoTo 2015. * **R programming language**. A good starting point is the R programming course at Coursera. * **Molecular biology**. Contact your local biologist. * **Robustness**. The book Wagner 2005 was a great inspiration for this work.

The standar format for logical regulatory networks is SBML-qual.

```
library(BoolNet)
library(BoolNetPerturb)
```

1.1 Robustness and plasticity in biological systems

Organisms develop in a changing world where they are exposed to intrinsic and extrinsic perturbations. Because of this perturbations, they need to be both resilient and adaptable, depending of the situation. This two behaviors coexist in all organisms, which suggests that there are common mechanism that underlie both robustness and plasticity.

Robustness is the capacity of an organism of maintaining its biological function in response to perturbations. A system is stable if it returns to the initial state after a perturbation, and plastic if it transitions to a new state. However, for studying robustness it is necessary to determine *what* function of the system is robust to *which* kind of perturbations[Wagner 2005].

1.2 Regulatory Networks

Regulatory Networks (RN) are a useful tool for studying the cellular behaivor and robustness of biological systems in response to different kinds of perturbations[Colomoto 2015]. RN integrate the available information of the molecular regulation to predict cellular level phenomena using a mathematical formalism. RN are deterministic dynamic systems. RN consist of nodes -that represent genes, proteins, or other biological processes- and edges -that represent the regulatory interactions among the nodes. Using this information, it is possible to construct functions that describe the state of the nodes depending on the state of its regulators. The value of the node represents wether the gene or protein is active or inactive in the biological system. The effect of the environment can be included in this models as input nodes.

The functions of the network are evaluated to obtain the attractors of the network. Attractors represent stable states in the dynamics of the network and have been related to cell types or biological processes like the cell cycle[Kaufman 1969, Azpeitia 2011, Albert 2014] [Figure 1]. RN let us simulate multiple types of perturbations depending in which part of the RN we alter. We can say that an *attractor* is stable to a perturbation if the RN returns to the same attractor, or plastic if it transitions to a different attractor. The robustness of the *system* is the result of the stability and plasticity of all its attractors. The robustness of the system can differ depending on the perturbation. In this way, robustness is a characteristic of the system that emerges from the interactions between the components of the system.

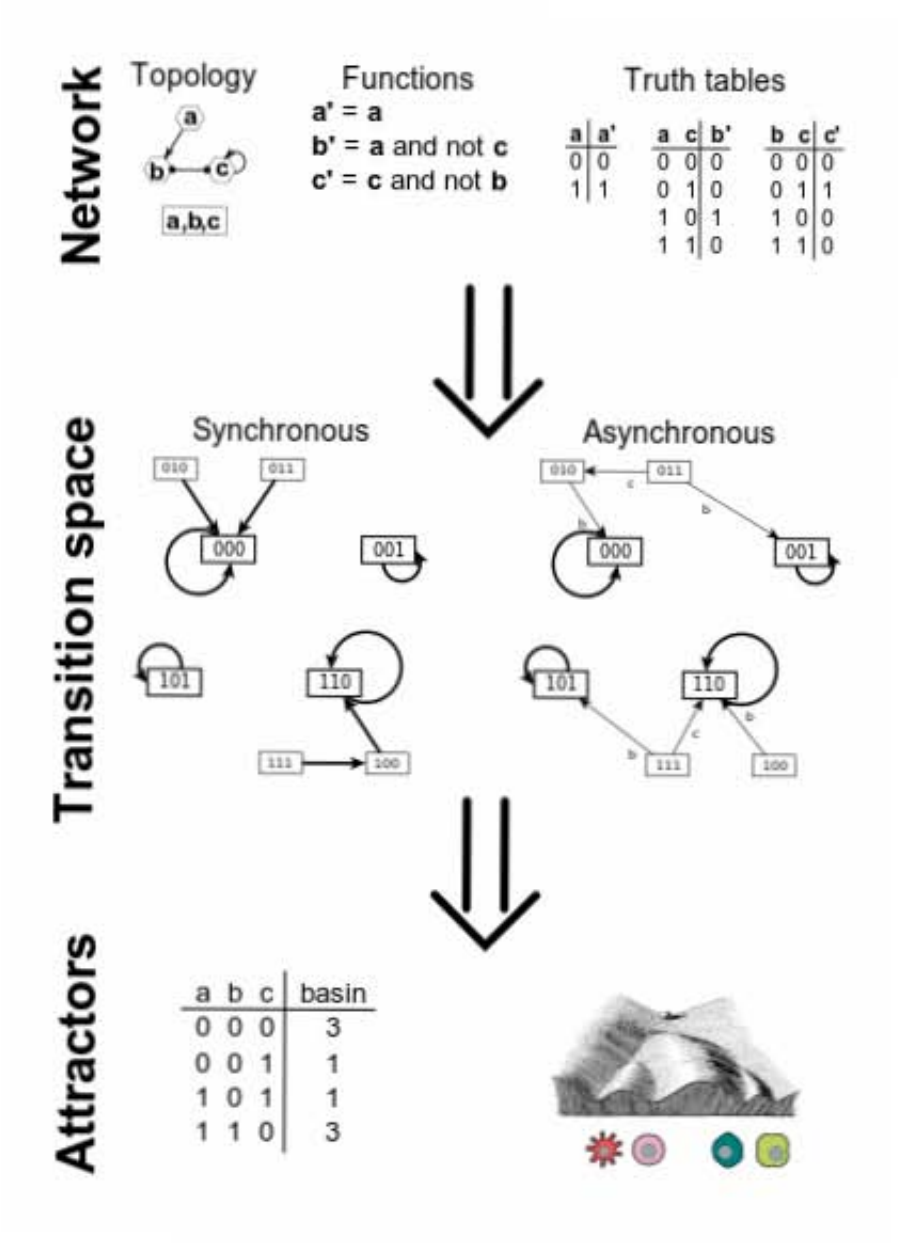


Figure 1: Boolean regulatory networks. A regulatory network consist of nodes, its interactions,

and the Boolean functions that regulate the value of each node. The state of each node can be updated using this functions, to obtain the transition space. The attractors of the network correspond to biological cell types.

1.3 Biological system: Th17/iTreg network

In this work we will use the Th17/iTreg regulatory network as an example. This network is a part of the CD4+ T cell regulatory network that has alredy been published and analysed using this methodology [Martinez-Sanchez 2015]. CD4 + T cells are fundamental for the adaptive immune response. They integrate the signals of the environment and differentiate from naive (Th0) cells into different cell types (Th17, iTreg, Th3, etc), which activate different parts of the immune system. In particular, Th17 cells have been associated with the inflammatory response and iTreg cells with the regulation of the inflammatory response.

CD4+ T cells begin as naïve Th0 cells, which do not express a transcription factor. These cells are activated by antigen presentation and differentiate depending in the cytokines in the environment. In the presence of IL-6 or IL-21 and TGFβ Th0 cells differentiate into Th17 cells and express RORγt, IL-21 and IL-17. In the presence of IL-2 and TGFβ Th0 cells differentiate into iTreg cells and express Foxp3 and TGFβ. There also exist Th3 cells, which are TGFβ+Foxp3-. These cytokines and transcription factors regulate each other and their relationships can be visualized a as graph[Zhu 2010, Carrier 2007].

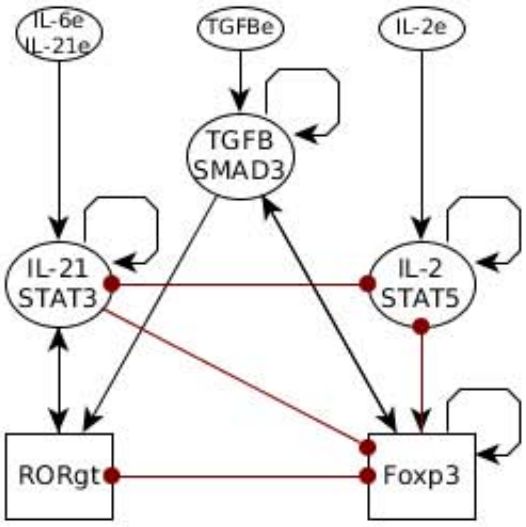


Figure 2. Th17/iTreg regulatory network

The Th17/iTreg network can be expressed as a set of boolean functions obtained from the known interactions among the cytokines and transcription factors. Cytokines are intrinsic if produced by the CD4+ T cell, and extrinsic if produced by other cells of the immune system. We will distinguish extrinsic cytokines by adding *e* at the end of the cytokine name.

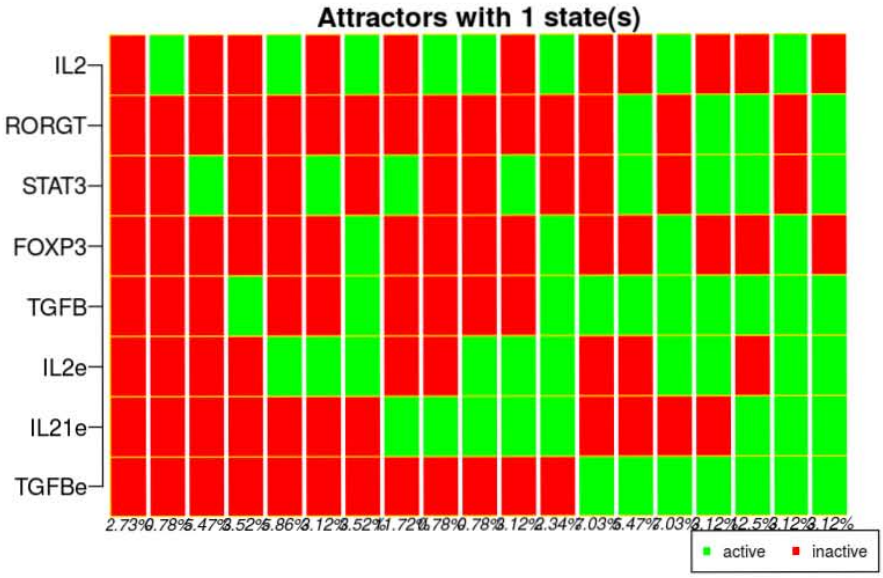
```
net <- loadNetwork("minTh17iTreg.txt")
net
```

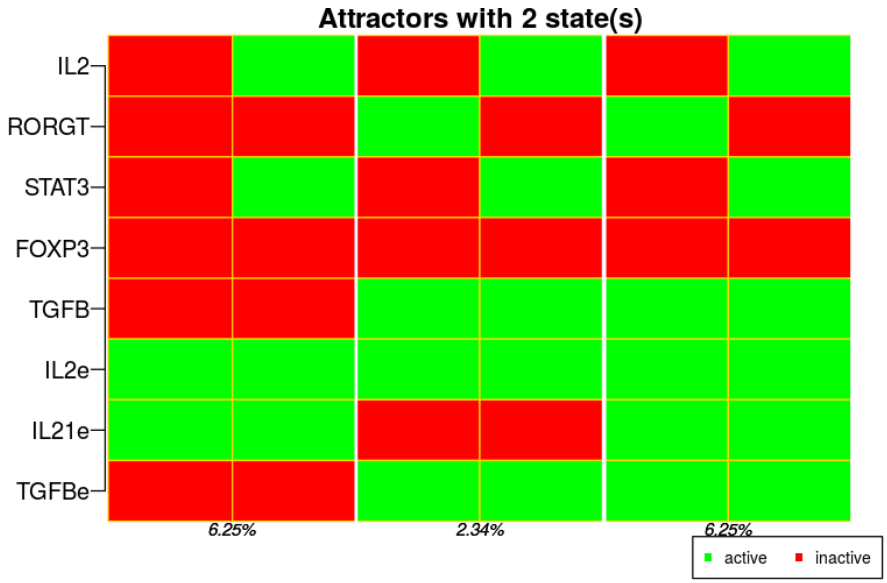
```
Boolean network with 8 genes

Involved genes:
IL2 RORGT STAT3 FOXP3 TGFB IL2e IL21e TGFBe

Transition functions:
IL2 = (IL2e | (IL2 &  ! FOXP3)) &  ! STAT3
RORGT = (STAT3 & TGFB) &  ! FOXP3
STAT3 = (IL21e | STAT3 | RORGT) &  ! IL2
FOXP3 = (IL2 & (TGFB | FOXP3)) &  ! (STAT3 | RORGT)
TGFB = TGFBe | ((TGFB | FOXP3) &  ! STAT3 )
IL2e = IL2e
IL21e = IL21e
TGFBe = TGFBe
```

2 Attractors of the network

As the state of the network is updated using the functions, the network will reach a previously visited state called an attractor. Attractors can be steady states or cycles. The set of states that lead to an attractor is called the basin of the attractor. Attractors represent cell types or biological processes. It is very important to verify that all the expected cell types appear in our attractors, if they are not present we might be missing interactions. It is also important to see if there are attractors that do not correspond to known cell types, as they may be predictions or show errors in the construction of the network.

In [3]:
```
attr <- getAttractors(net)
plotAttractors(attr)
```





2.1 Labels

When there are a lot of inputs, it is possible that many attractors correspond to a single cell type that can be found in different environments (we will discuss this further). We will consider that an attractor corresponds to a cell type if both the master transcription factor and characteristic cytokine are active.

```
In [4]: labels.rules <- data.frame(
            labels=c('Th0', 'Th17', 'Treg', 'Th3', 'RORGT+', 'FOXP3+'),
            rules= c('!(RORGT | FOXP3 | TGFB)', 'RORGT & STAT3',
                     'FOXP3 & TGFB', 'TGFB & ! (RORGT | FOXP3)',
                     'RORGT & ! STAT3', 'FOXP3 & ! TGFB')
            )
        labels.rules
```

labels	rules
Th0	!(RORGT | FOXP3 | TGFB)
Th17	RORGT & STAT3
Treg	FOXP3 & TGFB
Th3	TGFB & ! (RORGT | FOXP3)
RORGT+	RORGT & ! STAT3
FOXP3+	FOXP3 & ! TGFB

We can authomatically label using the **BoolNetPerturb** function **labelAttractors()** which takes a dataframe of the states to label, and a list of genes, labels and rules. If there is a cycle, we will label each state independently and join them with a "/".

```
In [5]: attr.df <- attractor2dataframe(attr)

        labels <- labelAttractors(attr, net$genes, labels.rules$labels,
                                  labels.rules$rules)
        attr.df$label <- sapply(labels, function(label) {
            paste(as.character(label), collapse='/')
        })
```

We can group the attractors according by label. We represent the Boolean attractors with decimal numbers to facilitate display.

```
In [6]: attractors.by.label <- data.frame(
            states = tapply(attr.df$involvedStates, attr.df$label, paste),
            basin = tapply(attr.df$basin, attr.df$label, sum)
        )
        attractors.by.label
```

	states	basin
RORGT+/Th3	178/181, 242/245	22
Th0	0 , 1 , 4 , 33 , 36 , 68 , 65 , 97 , 100	88
Th0/Th0	96/101	16
Th17	150, 182, 214, 246	62
Th3	16 , 144	27
Treg	57 , 121, 185, 249	41

3 Function perturbations

Functions recapitulate the regulatory interactions and determine the dynamic of the Boolean regulatory network. They are limited by the topology, as nodes can only be directly influeced by their regulators. The changes in the functions of a network can be associated with multiple biological phenomena. Knock out and over expresion experiments, environmental factors, evolution, epigenetics and the intrinsic flexibility of the regulatory mechanisms all alter the iteractions of the regulatory network of a biological system.

3.1 Knock-out and over-expression

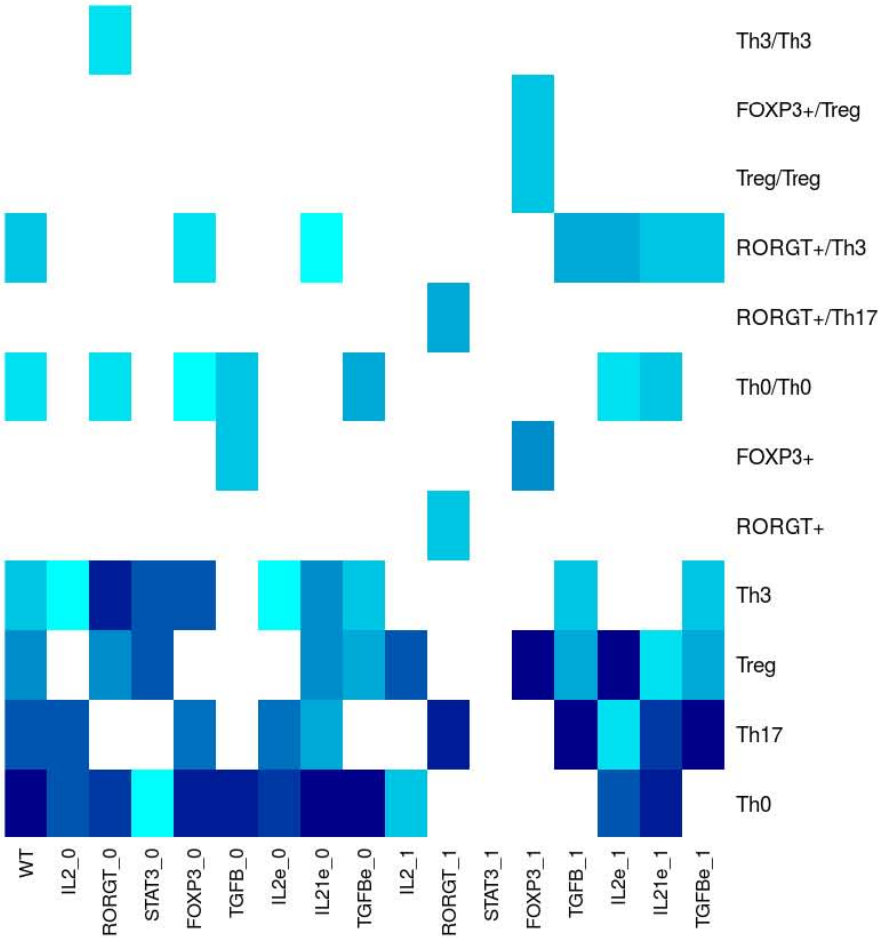
One possible kind of perturbation it to fix the value of the regulatory function to 0 or 1. The perturbation is equivalent to a knock-out or over-expression experiment. In this way it is possible to validate the model against known mutants. It can also be used to simulate the effect of conditional mutants of proteins that are hard to study in the wet lab, as the functions of the model only represent the effect of the mutation in the specific system. This can be useful if mutating the target protein is letal, as it can predict the effect of technically complicated conditional mutants.

We can use the **BoolNetPerturb** function **perturbNetworkFixedNodes()** to obtain the attractors and basins of the different fixed networks. By default, this method obtains all the single node knockouts and overexpressions. The function returns a dataframe where the rownames are the states and each column corresponds to the basin size of a fixed network. If the attractor cannot be found in a network it returns NA.

```
In [7]: # Obtain mutants
        mutants <- perturbNetworkFixedNodes(net)
```

This table can be labeled, ordered and plotted to study all single node mutants of the network.

```
In [8]: # Label all mutants
        labels <- lapply(rownames(mutants), function(states) {
            states <- as.numeric(unlist(strsplit(states, "/")))
            label <- lapply(states, function(s) {
                s <- int2binState(s, net$genes)
                l <- labelState(s, net$genes, labels.rules$labels,
                                labels.rules$rules)
            })
            label <- paste(label, collapse='/')
        })
        mutants$label <- as.character(labels)

        #Group by label
        mutants[is.na(mutants)] <- 0 # NAs to 0
        mutants.by.label <- by(mutants[, 1:(length(mutants)-1)],
                               mutants$label, colSums) #colSums by label
        mutants.by.label <- t(sapply(mutants.by.label,
                                     function(label) label ))
                                         #simplify and transpose

        #Order
        colnames(mutants.by.label) <- lapply(colnames(mutants.by.label),
                                             function(s) {
            unlist(strsplit(s, split='.', fixed=TRUE))[1]
        })
        mutants.by.label <- mutants.by.label[ c(
            'Th0', 'Th17', 'Treg', 'Th3', 'RORGT+', 'FOXP3+',
            'Th0/Th0', 'RORGT+/Th17', 'RORGT+/Th3',
            'Treg/Treg', 'FOXP3+/Treg', 'Th3/Th3'
        ) , ]

        #Plot
        mutants.by.label.matrix <- data.matrix(mutants.by.label) #convert to matrix
        mutants.by.label.matrix[mutants.by.label.matrix==0] <- NA #replace 0s for N
        colfunc <- colorRampPalette(c("cyan", "darkblue")) #color scale
        mutants.heatmap <- heatmap(mutants.by.label.matrix,  #heatmap
                                   Rowv=NA, Colv=NA,
                                   col= colfunc(10),
                                   #col=brewer.pal(3,"Blues"),
                                   scale="column", margins=c(5,10))
```



3.2 Fixed environments

The state of a network usually depends in external factors -that can be modeled as inputs. Fixing the value of the functions can also be used to study the effect of the environment in the differentiation and robustness of different cell types. Most cell-types are higly dependent on the cytokines in the environment. This can be simulated by fixing the values of the inputs of the network according to the different environments [Monteiro 2015].

```
In [9]: environments<- list(
            label=c('All', 'pro-Th0', 'pro-Th17', 'pro-iTreg'),
            nodes=list( c(NA),c('IL2e', 'IL21e', 'TGFBe'),
                        c('IL2e', 'IL21e', 'TGFBe'),
                        c('IL2e', 'IL21e', 'TGFBe')  ),
            values=list( c(NA, NA, NA), c(0, 0, 0),
                         c(0, 1, 1), c(1, 0, 1) )
        )

        environments.df <- data.frame( environments$values )
```

```
names(environments.df) <- environments$label
row.names(environments.df) <- c('IL2e', 'IL21e', 'TGFBe')
environments.df
```

	All	pro-Th0	pro-Th17	pro-iTreg
IL2e	NA	0	0	1
IL21e	NA	0	1	0
TGFBe	NA	0	1	1

The **BoolNetPerturb** function **perturbNetworkFixedNodes()** can simulate multiple fixed genes if we pass them as a vector inside a list.

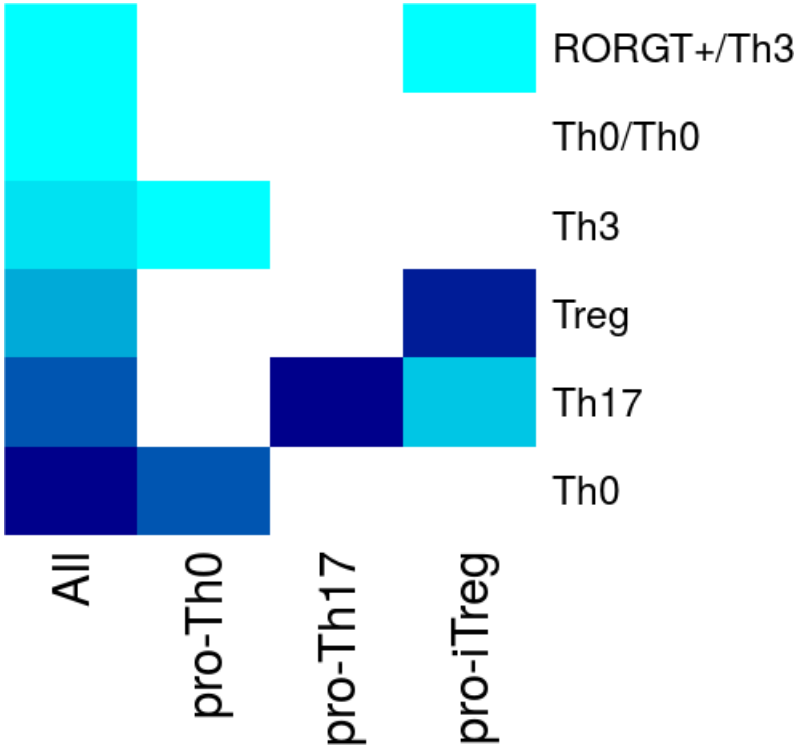
```
In [10]: # Simulate environments
         env.attr <- perturbNetworkFixedNodes(net, environments$nodes,
                                 environments$value, environments$label)
         #env.attr
```

This table can be labeled, ordered and plotted to study all the environments of the network.

```
In [11]: # label attractors
         env.attr$label <- unlist(lapply(rownames(env.attr),
                                         function(states) {
             states <- as.numeric(unlist(strsplit(states, "/")))
             label <- lapply(states, function(s) {
                 s <- int2binState(s, net$genes)
                 l <- labelState(s, net$genes, labels.rules$labels,
                                 labels.rules$rules)
             })
             label <- paste(label, collapse='/')
         }))

         # group by label
         env.attr[is.na(env.attr)] <- 0 # NAs to 0
         env.attr.by.label <- by(env.attr[, 1:(length(env.attr)-1)],
                                 env.attr$label, colSums) #colSums by label
         env.attr.by.label <- t(sapply(env.attr.by.label,
                        function(label) label )) #simplify and transpose
         #env.attr.by.label

         # rename and order
         colnames(env.attr.by.label) <- lapply(colnames(env.attr.by.label),
             function(s) {unlist(strsplit(s, split='.', fixed=TRUE))[1]})
         env.attr.by.label <- env.attr.by.label[ c(
             'Th0', 'Th17', 'Treg', 'Th3', 'Th0/Th0', 'RORGT+/Th3') , ]
         env.attr.by.label

         # plot
         env.attr.by.label.matrix <- data.matrix(env.attr.by.label) #convert to mat
         env.attr.by.label.matrix[env.attr.by.label.matrix==0] <- NA #replace 0s fo
```

```
colfunc <- colorRampPalette(c("cyan", "darkblue")) #color scale
env.attr.heatmap <- heatmap(env.attr.by.label.matrix,  #heatmap
                            Rowv=NA, Colv=NA,
                            col= colfunc(10),
                            scale="column", margins=c(25,25))
```

	All	pro-Th0	pro-Th17	pro-iTreg
Th0	88	23	0	0
Th17	62	0	32	8
Treg	41	0	0	18
Th3	27	9	0	0
Th0/Th0	16	0	0	0
RORGT+/Th3	22	0	0	6



3.3 Truth table

Trough evolution the regulatory interactions of an organism can change. This changes are not always loss-of-function mutations or over-expressions, they can also be smaller changes in the regulatory function. For example, the regulatory sequence of a gene can be altered, two independent regulators can become synergetic, or a positive interaction can become negative. However, this changes can also occur during the life of an organism: changes in the epigenetic marks, disordered domain proteins, and alternate spliccing can alter the regulatory functions[**?**]. It is possible that there are mistakes in the construction of the network when the fine regulatory logic is uncertain. It is possible to simulate this mutants by altering the truth table of the function, partialy

changing its regulatory logic. This partial changes in the functions have also been used to study the evolvability of biological systems, as the regulation of the gene expression can be mediated by changes in the promotors.

To do this we will use truth tables. Truth tables are a representation of Boolean functions. They have one column for each input variable, and one final column for all of the possible results of the logical operation that the table is meant to represent.

For example, the function: > C = A and not B

Can be represented as:

A	B	C
0	0	0
0	1	0
1	0	1
1	1	0

We can perturb this table by changing one of the values of the truth table.

A	B	C*
0	0	0
0	1	**1**
1	0	1
1	1	0

This new table corresponds to the function:

C = A xor B

We can simulate perturbations in the truth table using the **BoolNet** function **perturbNetwork()** and compare this results with the attractors of our WT network. Here we show the difference between the two sets of attractors. As **perturbNetwork()** randomly changes a value of the table it is a good idea to repeat the experiment multiple times (see BoolNet package vignate for more information). Here we show the perturbed states of the network after ten experiments.

```
In [12]: perturb.truth.table.differences <- function(net) {
             perturbed.net <- perturbNetwork(net, perturb="functions")
             pertubed.attr <- getAttractors(perturbed.net)
             pertubed.attr.df <- attractor2dataframe(pertubed.attr)
             #pertubed.attr.df
             setdiff(attr.df$involvedStates, pertubed.attr.df$involvedStates)
             }

         replicate(10, perturb.truth.table.differences(net))
```

1. '150'
2. '182'
3. '214'

4. '246'
5. '178/181'
6. '242/245'

4 Updating perturbations

The effect of the order in which the functions are evaluated can also be studied. Studying the robustness of the updating schema is useful for predicting the effect of temporal differences and developmental noise in the dynamic regulation of different proteins.

An other possible perturbation of the dynamic is to alter the sucessor states. Boolean regulatory networks are deterministic, the state in this time step determines the state in the next time step. However, it is possible to alter the successor, changing the transition graph. This can alter -or not- the trajectory of the simulation and change the attractor. This is equivalent to developmental noise in the differentiation process of a cell.

4.1 Synchronous vs asynchronous

In a discrete regulatory network the value of a node n in $t + 1$ is a function of the values of its regulators in the time $t. However, we can update the value of the nodes in different ways. The two main schemas for updating are synchronous -where all functions are evaluated at the same time- or asynchronous -where each function is evaluated independently. If synchronous updates are used each state the transition space has only one successor. However, if asynchronous updates are used each state the transition space can have more than one successor. Complex attractors depend on the updating policy and are harder and more expensive to compute in the asynchronous case.

Biologicaly speaking, synchronous updates supose that all the processes happen at exactly the same time, while asynchronous updates supose that the processes do not. If a process is faster than an other, or if there is a lag in its regulation, it will affect the dynamic of the regulatory logic. While most biological regulatory functions are asynchronous, it is easier to study the synchronous schema.

Lets compute the synchronous and asynchronous attractors and see the number of attractors each method found.

```
In [13]: attr.sync <- getAttractors(net)
         attr.async <- getAttractors(net, type="asynchronous")
         length(attr.sync$attractors)
         length(attr.async$attractors)
```

22
9

The asynchronous method returns less attractors. All the asynchronous attractors are also in the synchronous attractors, but the reverse is not true. In particular, most cycles tend to dissapear, as they are not robust to the update schema. Synchronous updates are deterministic, this means each state has only one sucessor. However, in asynchoronous updates, states can have more than one sucessor, which makes it hard to determine the basin size. This can complicate the analysis of the system and add computational time. It is for this reason that synchronous updating is

usually used. Asynchronous updates usually result in less attractors, however, all the attractors present in the asynchronous update will be present in the synchronous. It is important to check the asynchronous attractors as complex attractors -specially cyclic attractors- are not robust to the update method and may not occur in biological systems, which tend to be asynchronous.

4.2 Transition table

The trajectory of the regulatory network can be expressed as a transition table, where each state is followed by an other according with the differentiation functions. Perturbing the transitions between states can show the robustness to noise in the dynamic trajectory in the system.

We can do simulate this perturbations in the transitions using the **BoolNet** function **perturbNetwork()**. As perturbNetwork() randomly changes the transition table it is a good idea to repeat the experiment multiple times. Here we show the perturbed states of the network after ten experiments.

```
In [14]: perturb.transition.table.differences <- function(net) {
             perturbed.net <- perturbNetwork(net, perturb="transitions",
                                             numStates=10)
             pertubed.attr <- getAttractors(perturbed.net)
             pertubed.attr.df <- attractor2dataframe(pertubed.attr)
             #pertubed.attr.df
             setdiff(attr.df$involvedStates,pertubed.attr.df$involvedStates)
             }

         replicate(10, perturb.transition.table.differences(net))
```

1. '144'
2. '246'

5 States-and-Trajectories

Until now we have discused perturbations that occur in the functions or during evaluation of the regulatory network. However, once the attractors have been reached, they are subjected to other perturbations in the state of the system. This perturbations do not alter the wiring or updating of the network, but its state. This perturbations are transient, as the functions remain the same, it is only the state that is perturbed for a certain time, affecting its trajectory.

Biologicaly, this is equivalent to signals of the environment or intrinsic process of the cell that change the expression of an element for a certain time. For example, most drugs change the microenvironment of the organism while they are ingested, but once the treatment ends the perturbation ends. An other example of transient perturbations in biological systems is stochastic noise.

Lets take Th0 attractor [0,0,0,0,0, 0,0,0] and expose it to a Th17 environment for one time step.

```
In [15]: state <- 0
         new.traj <- perturbPathToAttractor(
                         int2binState(state,net$genes), net,
                         c('IL2e', 'IL21e', 'TGFBe'), c(0,1,1),
                         time=1, returnTrajectory = TRUE)
         new.traj
```

	IL2	RORGT	STAT3	FOXP3	TGFB	IL2e	IL21e	TGFBe
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	1	1
3	0	0	1	0	1	0	0	0
4	0	1	1	0	0	0	0	0
5	0	0	1	0	0	0	0	0

As we can see, the resulting attractor is different (0,1,0,0,0, 0,0,0) but the resulting label is the same (Th0).

A question we can ask ourselves is if an attractor is more sensible to perturbations in a particular node, and what transitions do these perturbations cause.

Lets take the Th0 0 attractor again and perturb one by one all its nodes for one time step.

```
In [16]: state.int <- 0
         label <- "Th0"
         state <- int2binState(state.int, net$genes)

         # perturb all nodes for time = 1
         perturbations <- sapply(names(state), function(node) {
             new.value <- as.integer(!state[[node]])
             new.state <- perturbPathToAttractor(state, net,
                          node, new.value, time=1)
             new.state <- new.state$attractors[[1]]$involvedStates
             new.label <- labelState(int2binState(new.state, net$genes),
                                 net$genes, labels.rules$labels,
                                 labels.rules$rules )
             c(state.int, label, node, new.value, new.state, new.label)
         })
         # clean data and rename columns
         perturbations <- t(perturbations)
         colnames(perturbations) <- c("ini.state", "ini.attr",
                                      "pert.node", "pert.value",
                                      "mod.state", "mod.attr")
         perturbations
```

	ini.state	ini.attr	pert.node	pert.value	mod.state	mod.attr
IL2	0	Th0	IL2	1	1	Th0
RORGT	0	Th0	RORGT	1	4	Th0
STAT3	0	Th0	STAT3	1	4	Th0
FOXP3	0	Th0	FOXP3	1	16	Th3
TGFB	0	Th0	TGFB	1	16	Th3
IL2e	0	Th0	IL2e	1	1	Th0
IL21e	0	Th0	IL21e	1	4	Th0
TGFBe	0	Th0	TGFBe	1	16	Th3

This table shows us all the possible one node perturbations for a state and their resulting attractors and labels. As we can see the Th0 attractor is very sensible to perturbations. However, most of the perturbations end in Th0 attractors, only a few end in Th3 attractors.

Using this method we can determine which cell types are more robust to perturbations and which are more plastic to perturbations. We can also determine which nodes are more important to the plasticity of the system, as they tend to cause more transition. It is also possible to study wether the length of the perturbations affects the transitions of the system.

6 Conclusions

RN are a powerful tool for studying biological systems. Using this modeling tool it is possible to recover not only the cell types, but also to study their robustness. RN let us realize global studies that show the properties of the system. This is particularly important when studying system properties like robustness. Furthermore, once we have a globa study it is possible to determine the roles of specific nodes in the dynamic behaivor of the whole system, finding targets for future research in the wet lab.

Models are representations of reality, this means they need to be carefully verified to make sure their predictions are relevant. To verify the model it is important to take into account: * Congruence with biological data, including mutants. * Avoid overfitting, make sure that small mistakes won't affect the model to much * Consider the limitations of the modeling tool and why this limitations create artifacts.

An important consideration is to define the biological functions that are being studied, and to determine what constitutes stability, plasticity and robustness for them. To better compare this properties between systems ee need to create quatitative measures for plasticity. Boolean networks might be useful, as they integrate levels to create a system approach, and it is possible to do sample most of the system. In the future it will be important to understand the general properties from random and biological networks. Depending on the question that needs to be adress is the methodology you use.

Target	Perturbation	Biological equivalent
Function	Fixed functions	Knock-out and over expressions, permanent changes in environment
	Transition table	Misconstruction of the network, small changes in regulation, evolvability
Updating	Dynamic	Time and hierarchy of biological processes.
	State transitions	Transient biological behaivor.
Attractor	State of nodes	Temporal changes in expression, transient environmental signals.
	Stochastic	Biological stochastic processes.

We hope this software is useful to both experimental and theoretical biologists who wish to study robustness.

7 References

- Albert, R., & Thakar, J. (2014). Boolean modeling: a logic-based dynamic approach for understanding signaling and regulatory networks and for making useful predictions. Wiley Interdisciplinary Reviews: Systems Biology and Medicine, 6(5), 353–369. https://doi.org/10.1002/wsbm.1273

- Azpeitia, E., Benítez, M., Padilla-Longoria, P., Espinosa-Soto, C., & Alvarez-Buylla, E. R. (2011). Dynamic network-based epistasis analysis: boolean examples. Frontiers in Plant Science, 2, 92. https://doi.org/10.3389/fpls.2011.00092
- Carrier, Y., & Yuan, J. (2007). Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-β T cell-transgenic mice. The Journal of . . . , 7(5), 335–336. https://doi.org/10.1038/nmeth0510-335
- Kaplan D, Glass L (1995) Understanding nonlinear dynamics. New York: Springer.,
- Kauffman, S. A. (1969). Metabolic stability and epigenesis in randomly constructed genetic nets. Journal of Theoretical Biology, 22(3), 437–467. https://doi.org/10.1016/0022-5193(69)90015-0
- Martinez-Sanchez, M. E., Mendoza, L., Villarreal, C., & Alvarez-Buylla, E. R. (2015). A minimal regulatory network of extrinsic and intrinsic factors recovers observed patterns of CD4+ T cell differentiation and plasticity. PLoS Comput Biol, 11(6), e1004324.http://dx.doi.org/10.1371/journal.pcbi.1004324
- Monteiro, P. T., Mu, C., Kestler, H. a., Thieffry, D., Xenarios, I., Saez-Rodriguez, J., … Chaouiya, C. (2015). Cooperative development of logical modelling standards and tools with CoLoMoTo. Bioinformatics, 31(January), 1154–1159. https://doi.org/10.1093/bioinformatics/btv013
- Müssel, C., Hopfensitz, M., Kestler, H. A., Christoph, M., Hopfensitz, M., & Kestler, H. A. (2010). BoolNet package vignette. Tutorial, 1–34.
- Wagner, A. (2005). Robustness, evolvability, and neutrality. FEBS letters, 579(8), 1772-1778.
- Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations (*). Annual Review of Immunology, 28, 445–89. https://doi.org/10.1146/annurev-immunol-030409-101212

Discusión y conclusiones

Las células T CD4+ se desarrollan en un entorno que cambia constantemente. Como parte del sistema inmune, estas células deben enfrentarse y colaborar con el microbioma, de tal forma que sus circunstancias cambian constantemente. Además la misma célula esta sujeta a ruido en los procesos celulares. Para adecuarse a este entorno cambiante, los seres vivos han desarrollado mecanismos que les permiten ser tanto estables como plásticos dependiendo de las circunstancias. El entender los mecanismos internos que subyacen estos comportamientos es importante no solo para explicas el desarrollo de las células T CD4+, sino para entender la emergencia de la robustez en los sistemas complejos. En el presente trabajo hemos estudiado los mecanismos que subyacen la diferenciación y la plasticidad de las células T CD4+. Para hacer esto hemos estudiado tanto el sistema biológico como las propiedades generales de las redes de regulación Booleanas.

La diferenciación y plasticidad de las células T CD4 + surge de la retroalimentación entre múltiples niveles de regulación: transcripcional, vías de señalización y el microambiente. El estudio de las redes de regulación como un sistema dinámico nos permite entender cómo las interacciones entre los componentes, la topología de la red y las funciones dinámicas de los nodos dan lugar al comportamiento biológico. Además, estas redes nos permiten estudiar la robustez del sistema inmune, recuperando la plasticidad de las células T CD4+ en respuesta al microambiente y a las perturbaciones transitorias de los componentes del sistema.

Los modelos aquí presentados nos permiten determinar que factores son necesarios y suficientes para capturar la determinación celular de las células T CD4+. Cabe señalar que los factores transcripcionales maestros no son suficientes, es necesario incluir la retroalimentación y dinámica cooperativa con las vías de señalización y las citocinas tanto producidas por el mismo linfocito como aquellas provenientes de otras células. De esta manera, es posible recuperar las configuraciones observadas para células: Th0, Th1, Th2, Th17, Tfh, Th9, iTreg, Tr1 y Th3. En particular, cabe destacar que el modelo es capaz de explicar la diferenciación de las células Tr1 (Foxp3-IL10+) y Th3 (Foxp3-TGFβ+), las cuales no están asociadas con algún factor transcripcional maestro pero son fundamentales para regular al sistema inmune y mantener la tolerancia del organismo. Además, el modelo recupera varios tipos celulares híbridos reguladores similares a Th1 y Th2, los cuales también han sido reportados. El que el modelo recupere estos tipos celulares muestra que la dife-

renciación no es necesariamente un proceso jerárquico, sino el resultado de una red de regulación multinivel.

Las citocinas en el microambiente restringen qué tipos de células y transiciones pueden alcanzar las células T CD4+. En un micro-ambiente que favorece un tipo de célula particular aumenta el tamaño de la cuenca de atracción, su estabilidad y transiciones hacia el tipo celular que se está favoreciendo, sin embargo, otras células T CD4 + pueden coexistir en este microambiente. Por ejemplo, en los entornos efectores como Th1, Th2 y Th17 coexisten células efectoras con células reguladoras. Por otro lado, la presencia de citocinas pro-reguladoras IL-10 y TGF-β inhibe la mayoría de las células efectoras, excepto Th17. Este hallazgo puede explicar la presencia de células Th17 en micro-ambientes regulatorios, mejorando nuestro entendimiento sobre la relación entre Th17 y iTreg y el papel bivalente de TGF-β tanto en la inflamación como en la regulación.

Dada la complejidad de los patrones de expresión de las células T CD4 + y las transiciones entre ellos, es difícil determinar si los tipos celulares corresponden a linajes o subtipos. El término linaje implica estabilidad y compromiso del fenotipo, independientemente de las alteraciones ambientales. Por otro lado, el término subtipo implica que, aunque la célula tiene un patrón de expresión especificado, depende de las señales extrínsecas para su mantenimiento. Los tipos de células Th1, Th2, Tfh, Tr1 y Th3 son estables ante alteraciones en las citocinas extrínsecas. Mientras tanto, las células Th17, iTreg y Th9 requieren TGF-βe, IL-2e o IL-4e, respectivamente. Sin embargo, todos los tipos celulares son sensibles a cambios en el entorno o perturbaciones transitorias dirigidas. De esta manera, no se puede decir que un tipo celular sea un linaje o un subtipo, mas bien, lo que existe es un continuo de estabilidad y plasticidad que se puede ver alterado en circunstancias específicas. Usando metodologías como las aquí presentadas, es posible estudiar el grado de dependencia de las señales extrínsecas y la estabilidad en respuesta a los cambios en el microambiente, lo cuál pueden proporcionar criterios más claros y objetivos para estudiar la robustez de las células T CD4+.

Las células T CD4+ son fundamentales para modular la respuesta inmune. Una vez diferenciadas, estas células activan o inhiben diferentes mecanismos de la respuesta inmune. Es por esto que las redes de regulación aquí presentadas no solo nos ayudan a entender el comportamiento de las células T CD4+, sino también nos dan valiosas intuiciones sobre el balance entre la inflamación y la regulación de la respuesta inmune. Los procesos inflamatorios son fundamentales para controlar a muchos de los retos inmunológicos, pero al mismo tiempo la inflamación crónica esta asociada con enfermedades como cáncer, alzheimer y síndrome metabólico.

La obesidad está fuertemente relacionada con la resistencia a la insulina, hiperinsulinemia, inflamación crónica y alteraciones en el comportamiento de las células T CD4+. Para estudiar la interacción entre el sistema inmune con el metabolismo es necesario integrar los datos de regulación molecular disponibles en modelos sistémicos dinámicos que permitan predecir los comportamientos a nivel celular. Como un primer caso de estudio nos centramos en el efecto de la hiperinsulinemia en las células T CD4+ en el tejido visceral adiposo obeso, ya que se conocen los mecanismos de regulación molecular por medio de los cuales la hiperinsulinemia afecta la producción de citocinas de las células T CD4+. Utilizando esta estrategia somos capaces de generar modelos dinámicos

que recuperan algunos de los comportamientos de los linfocitos T CD4+ en la obesidad asociada a inflamación crónica.

La hiperinsulinemia altera la dinámica de la red de regulación de las células T CD4+, y esto a su vez, modula la diferenciación celular y la plasticidad. En presencia de altos niveles de insulina, tipos celulares proinflamatorios como Th1 y Th17 se vuelven más estables a perturbaciones transitorias y sus tamaños de cuenca aumentan, células reguladoras como Tr1 se vuelven menos estables o desaparecen, mientras que las células productoras de TGFβno se ven afectadas. Como ya se vio previamente, en ambientes pro-Th1, pro-Th2 y pro-Th17 coexisten células efectoras y reguladoras, pero los altos de insulina afectan severamente a las células reguladoras, especialmente en un entorno pro-Th17, desplazando el equilibrio del sistema inmune hacia la inflamación. El modelo también resalta el papel bivalente de TGFβen la aparición del fenotipo Treg adiposo.

Las simulaciones resultantes proporcionan predicciones novedosas sobre el impacto del microambiente en la coexistencia de los diferentes tipos celulares. De esta manera, los modelos de redes Booleanas proporciona un marco formal para integrar los datos experimentales en el estudio de enfermedades inflamatorias complejas, realizando predicciones que pueden ser verificadas experimentalmente. La retroalimentación entre los modelos y los experimentos mejora nuestro entendimiento de los sistemas biológicos, permitiéndonos proponer estrategias para tratar las enfermedades complejas.

Los modelos son representaciones de la realidad, esto significa que necesitan ser cuidadosamente verificados para asegurarse de que sus predicciones son relevantes. Para verificar el modelo es importante tener en cuenta la congruencia con datos biológicos, evitar sobreajustes, y considerar las limitaciones de los datos y las herramienta de modelado. En el caso de las redes Booleanas existen varias metodologías que permiten validar el modelo, lo cuál le da mas fiabilidad al modelo propuesto. Al mismo tiempo, el modelo permite detectar áreas donde nuestro conocimiento es incompleto, detectar patrones sistémicos y realizar hipótesis que pueden ser comprobadas experimentalmente.

A lo largo de este trabajo destaca la importancia de las asas de retroalimentación, ya sea entre el sistema metabólico e inmune como dentro de las mismas células T CD4+. En la mayoría de los tipos celulares se pueden ver asas de retroalimentación positivas entre el factor transcripcional maestro a la citocina característica, al mismo tiempo que hay asas de retroalimentación negativa con otros tipos celulares. Las asas de retroalimentación son fundamentales para la robustez del sistema, y entenderlas no solo nos da intuiciones sobre el funcionamiento de las células T CD4+, sino también de las propiedades generales de los sistemas biológicos. Los modelos aquí presentados muestran que las redes de regulación no son jerárquicas y unidireccionales, sino altamente interrelacionadas con múltiples asas de retroalimentación entre los distintos niveles.

Las redes Booleanas nos permiten representar los reguladores de un sistema biológico y sus interacciones como la topología y las funciones de la red. Utilizando esta representación es posible predecir los patrones de expresión como los estados de la red, de tal forma que los atractores corresponden a los tipos celulares. Sin embargo, entender la relación entre la topología, las funciones y los atractores no es trivial, sobretodo cuando múltiples asas de retroalimentación conviven en una sola

red como es el caso de los sistemas biológicos. Varios conjuntos de funciones pueden corresponder a una sola topología, y un conjunto de atractores puede ser el resultado de varias topologías y conjuntos de funciones diferentes. Generalmente, las asas de retroalimentación determinan el número y tamaño de atractores de una red, sin embargo, cuando hay múltiples asas de retroalimentación, estas no son funcionales en todas las circunstancias.

Entender la relación entre la topología, las funciones dinámicas, y los circuitos funcionales es importante para entender la emergencia de los atractores de las redes Booleanas. Sin embargo, estudiar estos patrones en redes biológicas puede ser sumamente complicado. Una forma de abordar esta pregunta es comparar pequeñas vías de regulación. De esta manera podemos ver que son los circuitos funcionales de retroalimentación los que determinan las características de los atractores del sistema, en particular el número y tamaño de estos. Al comparar dos vías de regulación podemos ver que dos redes con topologías similares pueden dar atractores distintos, mientras que dos redes con topologías diferentes pueden dar atractores similares. Por otro lado, redes con el mismo circuitos funcional tienden a ser mas similares. Sin embargo, el patrón no es del todo claro y es necesario realizar mas estudios al respecto.

Dada la importancia de las redes Booleanas como herramientas en el estudio de los sistemas biológicos, es importante desarrollar paqueterías que permitan automatizar el análisis de las redes Booleanas. Para lograr esto es necesario definir la función biológica que se están estudiando y determinar qué es lo que constituye estabilidad, plasticidad y robustez en ese contexto. De esta manera es posible dar metodologías que sistemáticamente caractericen a los sistemas para obtener simulaciones confiables. El fijar las funciones como cero o uno permite simular mutantes de pérdida o ganancia de función y comparar con los mutantes disponibles experimentar. Fijar el valor de los nodos de entrada permite simular distintos microambientes. Alterar aleatoriamente las tablas de verdad y de transición permite estudiar el efecto de errores en la construcción de la red, pequeños cambios en la regulación y el ruido durante el desarrollo. Comparar diferentes esquemas de actualización permite estudiar el efecto del tiempo, constantes de decaimiento y jerarquía de los procesos biológicos en la dinámica del sistema. Perturbar transitoriamente el estado de los nodos permite estudiar la estabilidad del sistema ante cambios temporales en la expresión y señales ambientales transitorias. De esta manera, la metodología aquí presentada permiten estudiar la robustez de los sistemas biológicos a varios niveles en las redes booleanas, complementando las metodologías ya existentes.

Las células T CD4+ son un ejemplo de como los organismos requieren al mismo tiempo estabilidad y plasticidad. Estas células modulan la defensa del sistema inmune contra patógenos y al mismo tiempo mantienen la tolerancia del organismo, evitando el desarrollo de enfermedades autoinmunes. Más aun, las células T CD4+ son un ejemplo de como los organismos han desarrollado mecanismos para enfrentarse a entornos cambiantes. Tanto el medio ambiente como la regulación intrínseca presentan perturbaciones transitorias, por lo cuál es necesario tomar en cuenta estos fenómenos para entender a los sistemas biológicos. El desarrollar modelos que permitan estudiar

los mecanismos de regulación del sistema inmune capaces de generar comportamientos dinámicos robustos es importante para el tratamiento de enfermedades sistémicas.

Los modelos aquí presentados son principalmente redes de regulación mínimas. Aunque estos modelos recuperan varios comportamientos biológicos importantes aún carecen de señales y de la regulación de otras células que son fundamentales para entender completamente al sistema inmune. La principal limitación de este trabajo es que la red supone que la señal del TCR esta presente, sin incorporar explicitamente los diferentes componentes de esta vía de señalización e ignorando como la intensidad y duración de la activación del TCR afecta a las células T CD4+. Además, estas redes ignoran citocinas tales como IL-1, IL-22 y TNFα y factores transcripcionales como PU.1 y c-maf. En el caso del modelo de hiperinsulinemia el modelo reduce excesivamente el papel del metabolismo, esta red ignora el efecto del metabolismo celular y el papel de hormonas como leptina, adiponectina o hormonas sexuales, y tipos de células adicionales tales como adipocitos y macrófagos,que juegan un papel importante durante la obesidad asociada a la inflamación crónica. Modelos futuros deberan incorporar estos factores para caracterizar adecuadamente la red de regulación que subyace la diferenciación y plasticidad de los linfocitos T CD4+.

Al ser las redes presentadas aquí modelos Booleanos solo son capaces de expresar valores discretos. Esto es una limitante particularmente fuerte en el caso del TCR, TGFβe insulina, las cuales se saben que su comportamiento varia dependiendo de la fuerza y longitud de las señales. Estudios continuos, asíncronos o de longitud de señal pueden ser útiles para evaluar diferentes síndromes y tratamientos, modelando de manera mas realista la progresión de la respuesta inmune. El modelo aquí utilizado, todavía simplifica el microambiente, que es mucho más complejo in vivo. Por ejemplo, es interesante evaluar cómo la longitud y la intensidad de las pequeñas señales iniciales que se producen en respuesta a la naturaleza variable del entorno, con el tiempo dar lugar a importantes alteraciones asociadas con diferentes enfermedades. Estudios adicionales sobre el efecto de las señales transitorias en una versión asíncrona o continua de la red reguladora de células T CD4 + mínima y extendida, probablemente proporcionarán información importante sobre tales patrones temporales.

También será fundamental determinar el efecto de la simplificación del modelo en los comportamientos transitorios observados in vivo, ya que los retrasos en las vías de señalización pueden afectar a la heterogeneidad, la plasticidad y la dinámica de la población de células T CD4 +. La simplificación del modelo también puede ocultar algunas de las moléculas involucradas en el sistema, lo que complica la validación experimental.

Conclusiones

Las células T CD4+ modulan al sistema inmune para mantener la homeostasis del organismo ante retos inmunológicos cambiantes. Esto hace que para mantener la robustez del sistema, las células T CD4+ deban ser estables en ciertas circunstancias y plásticas en otras. En este trabajo presentamos la red de regulación molecular que subyace este comportamiento dinámico. La red recupera los patrones de diferenciación y plasticidad en distintos entornos, ademas de hacer predicciones que pueden ser comprobadas experimentalmente.

El como el sistema inmune se comunica con otros sistemas es una pregunta compleja. Aquí presentamos una propuesta de meto logia para integrar el efecto de la hiperinsulinemia en el síndrome metabólico sobre la diferenciación de los linfocitos T CD4+. El modelo resultante recupera algunos de los patrones que se observan en la inflamación crónica asociada a la obesidad. Este es un paso adelante en resolver la pregunta de como se integra el sistema inmune con el metabolismo.

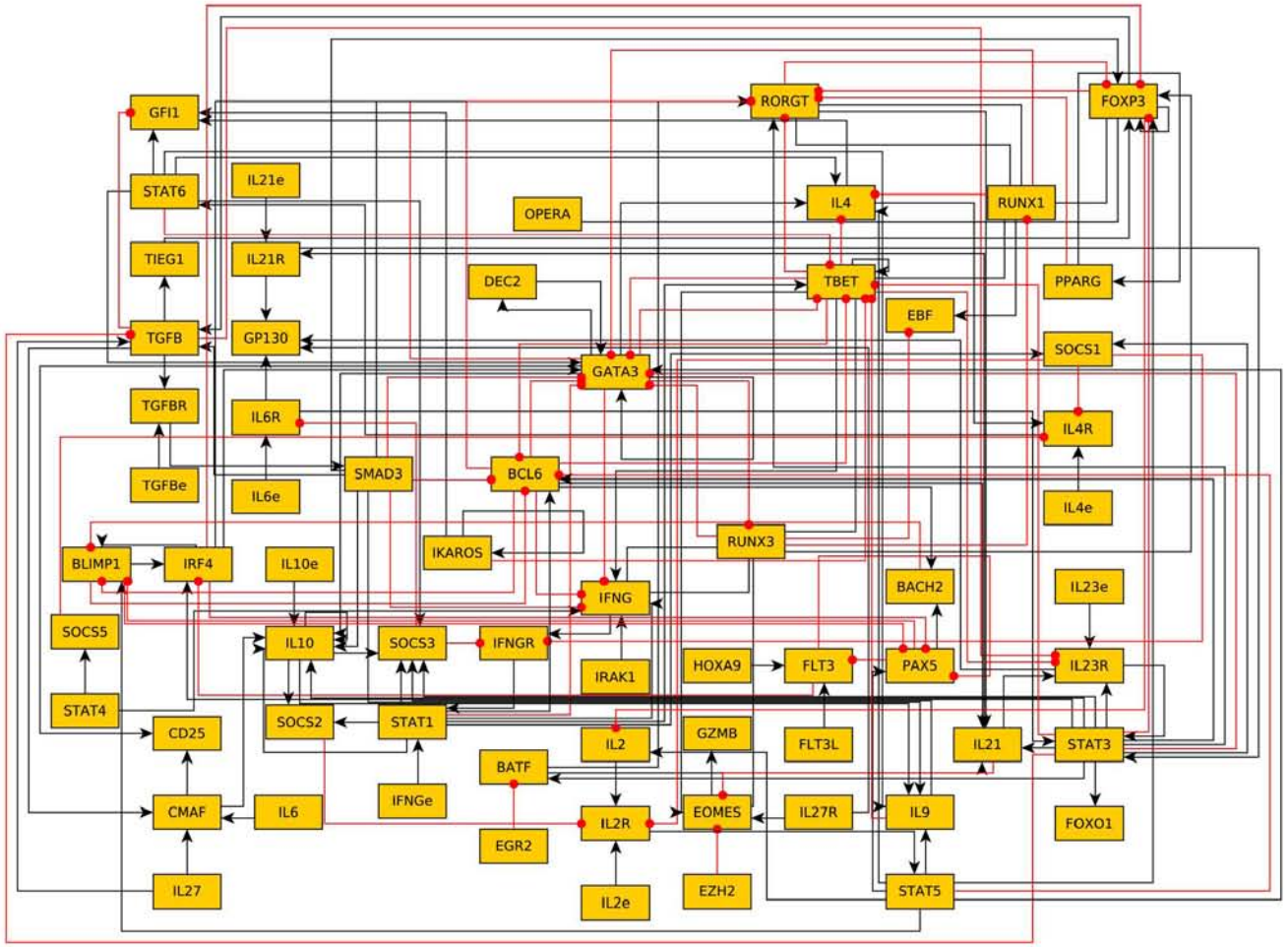
Al estudiar las redes biológicas se pueden ver patrones de asas de retroalimentación. Entender como el funcionamiento de estos circuitos modula a los sistemas biológicos es una pregunta importante no solo para las células T CD4+ sino para la biología de sistemas en general. Dada la complejidad de estudiar el papel de los circuitos funcionales en redes biológicas, estudiamos vías de regulación sencillas. De esta manera determinamos que son los circuitos funcionales, mas que la topología o las funciones, los que determinan la dinámica del sistema.

Tanto el entorno como la regulación interna de la célula están sujetas a perturbaciones, muchas de estas transitorias. Para ayudar al estudio del efecto de las perturbaciones en los sistemas dinámicos, presentamos una herramienta que permite realizar experimentos de simulación con perturbaciones en redes Booleanas.

El estudio de la red molecular que subyace la diferenciación de las células T CD4+ como un sistema dinámico nos permite entender cómo las interacciones entre los componentes de la red dan lugar al comportamiento biológico robusto del sistema inmune. Sin embargo, se necesitan más investigaciones teóricas y experimentales para comprender las células T CD4 +. A medida que mejoremos nuestra comprensión de estas células, será posible generar nuevas respuestas terapéuticas y mejorar nuestro entendimiento de las propiedad generales de los sistemas biológicos. Esperamos que los resultados y la metodología asociada sean útiles tanto para científicos experimentales como teóricos para simular, validar y analizar sistemas biológicos complejos.

Apéndice A

Supplementary: A Minimal Regulatory Network of Extrinsic and Intrinsic Factors Recovers Observed Patterns of CD4+ T Cell Differentiation and Plasticity


GFI1
STAT6
TIEG1
TGFB
TGFBR
TGFBe
BLIMP1
IRF4
SOCS5
STAT4
CD25
CMAF
IL27
IL21e
IL21R
GP130
IL6R
IL6e
IL10e
IL10
SOCS2
IL6
SMAD3
SOCS3
STAT1
IFNGe
IFNGR
IRAK1
BATF
EGR2
IKAROS
BCL6
OPERA
DEC2
GATA3
IFNG
IL2
IL2R
IL2e
RORGT
IL4
TBET
EBF
RUNX3
HOXA9
FLT3
FLT3L
GZMB
EOMES
EZH2
IL27R
PAX5
BACH2
IL21
IL9
STAT5
FOXP3
RUNX1
PPARG
SOCS1
IL4R
IL4e
IL23e
IL23R
STAT3
FOXO1

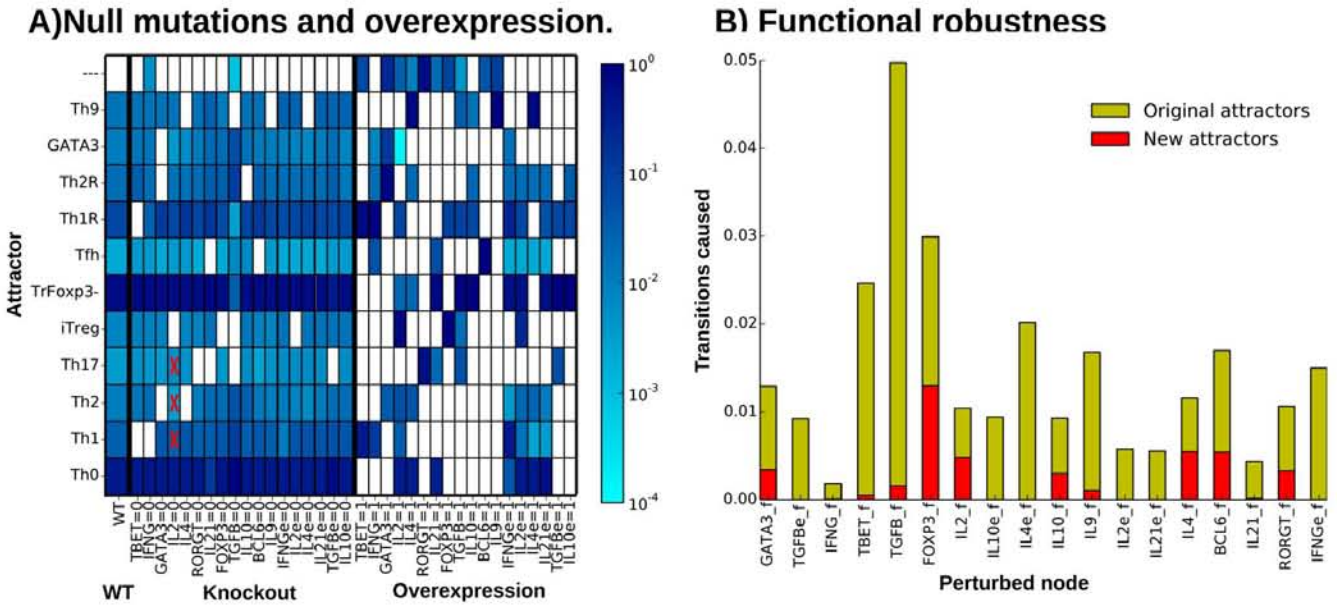


Figure S4. Validation of the T CD4+ lymphocyte trancriptional-signaling regulatory network.

(A) To validate the T CD4+ T-SRN model we simulated loss of function or null mutations (KO) and overexpression experiments and compared with available experimental data. The value of the nodes was set to "0" for simulations of loss-of-function or knockout experiments, and to "1" for overexpression. The color in corresponds to the basin size of each attractor in logaritmic scale. '---' represents attractors that were not attained in the original wild type (WT) network. The attractors marked with (red) "X" correspond to incorrect predictions.

(B) To verify the construction of the functions and the structural properties of the model we performed a robustness analysis altering the update rules. Networks with perturbed functions of the T CD4+ T-SRN were generated to test the robustness of structural properties of the networks to noise, mis-measurements and incorrect interpretation of the data. After altering one of the functions of the network, 1.389% of the possible initial states changed their final attractor (yellow), and only 0.219% of the possible initial states arrived to an attractor not in the original network (red).

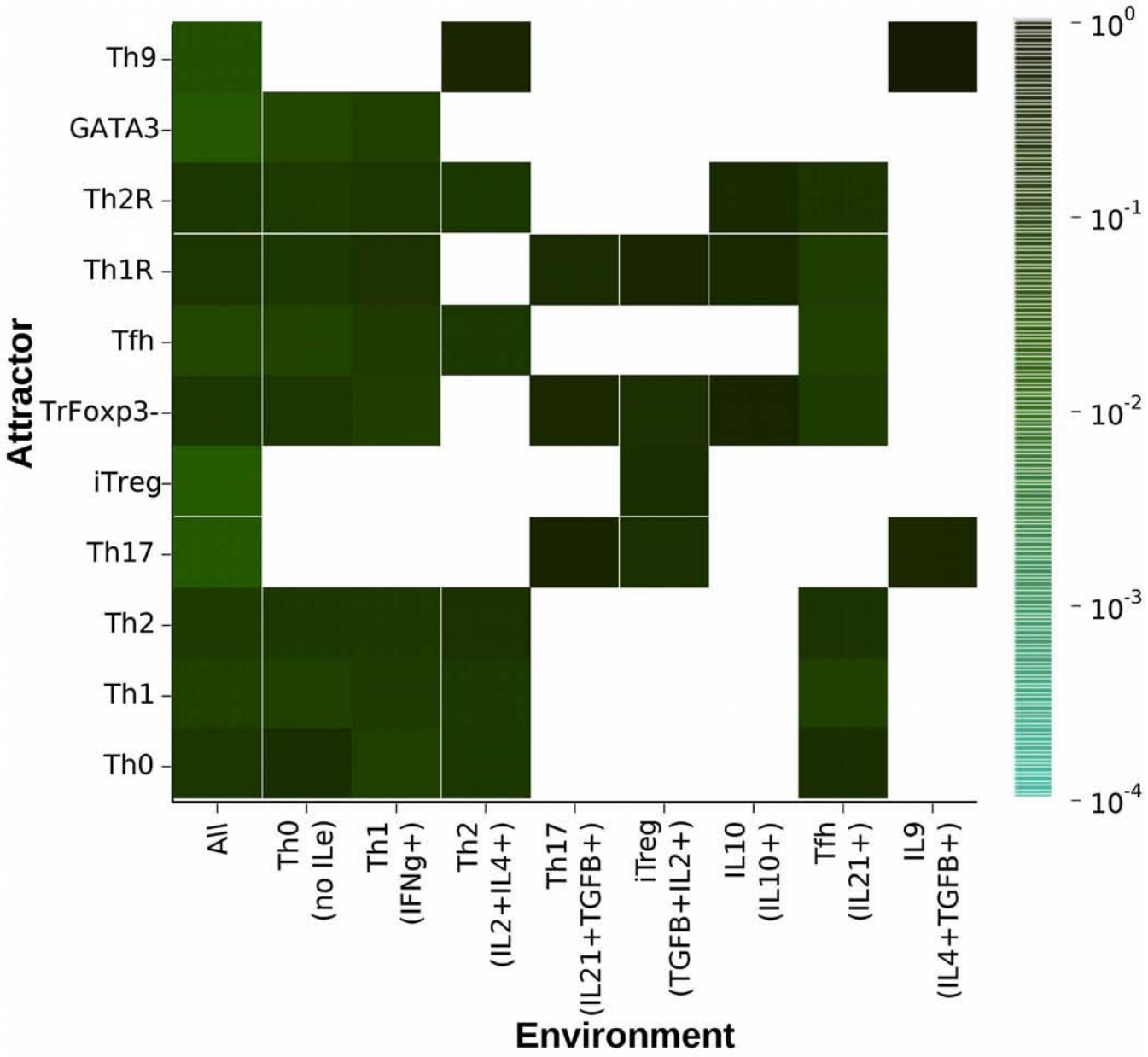


Figure S5. Effect of the environment in the stability of the T CD4+ lymphocyte trancriptional-signaling regulatory network.
The values of the extrinsic signals of the T CD4+ T-SRN were fixed in accordance to different polarizing environments. Each attractor was transiently perturbed and the proportion of transitions that stayed in the same cell type was ploted in logarithmic scale. The environment studied where: combinations of all extrinsic cytokines, Th0 has no extrinsic cytokines, Th1 has IFNγe, Th2 IL-4e and IL-2e, Th17 IL-21e and TGFβe, iTreg has TGFβe and IL-2e, IL-10 has IL-10e, Tfh has IL-21e, and Th9 has IL-4e and TGFβe.

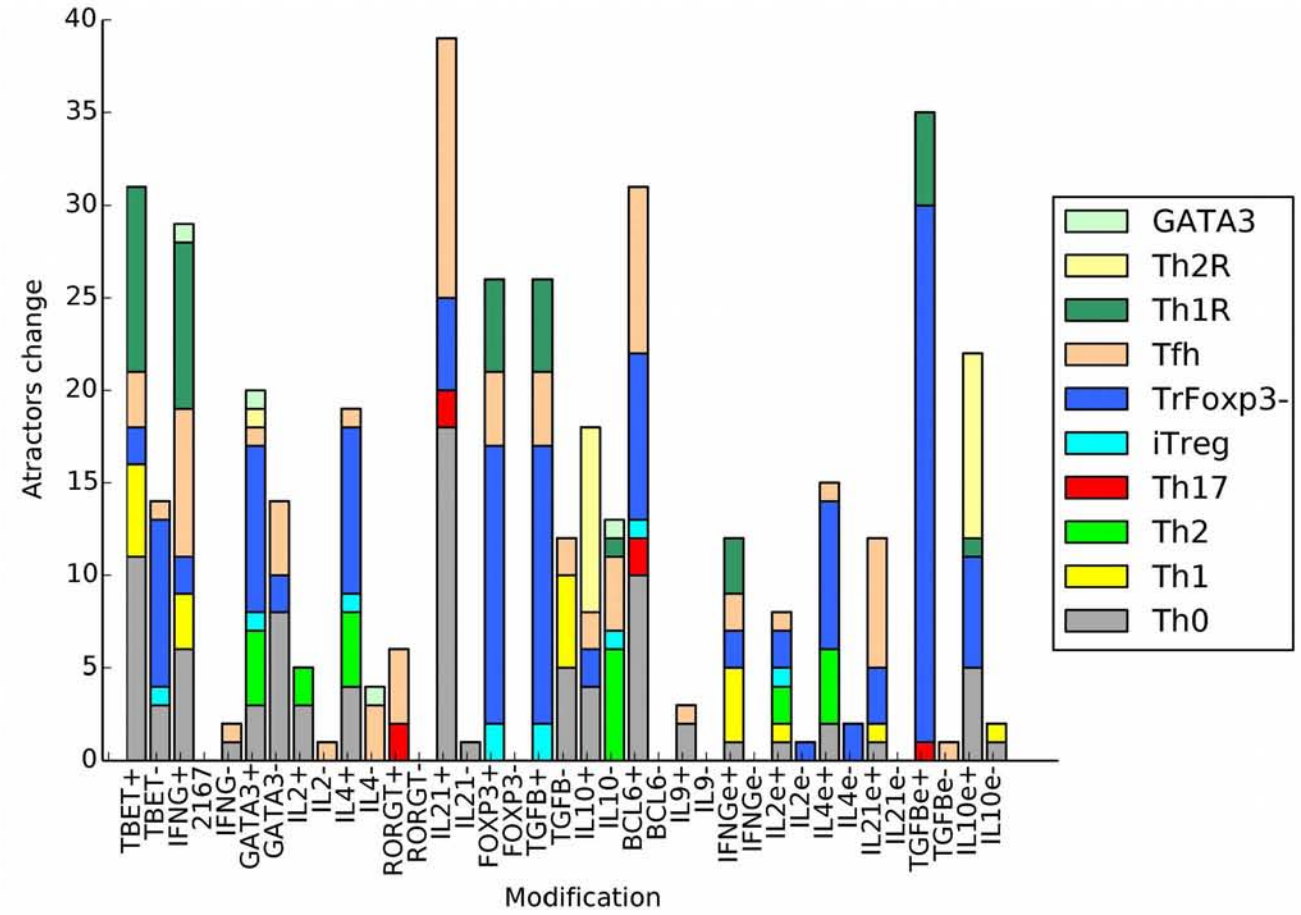


Figure S6. Effect of transient perturbations in the state of the nodes of the T CD4+ lymphocyte trancriptional-signaling regulatory network.
Number of transitions to an attractor in response to transient perturbations in the value of each node. The states of the node was perturbed during one time step from 1 to 0 (-), or 0 to 1 (+), depending on its state in the original attractor.

CD4+ T cell interactions

Source	interaction	Target	Reference	Notes
AKTHIGH	inhibition	IL10	Han2014	
AP1	activation	FOXP3	Delacher2014	
AP1	activation	IL10	Ouyang2011	
BACH2	inhibition	BLIMP1	Nutt Et Al., 2011, Ochiai Et Al., 2006	
BATF	increase	IL17	Schraml2009	Specific requirement, indepe
BATF	increase	IL21	Schraml2009, Li2012, Glasmacher2012	
BATF	activation	RORGT	Schraml2009ap-1	Ap1 family
BCL3	increase	GATA3	Das2001, Kusam2003	Ikb family member
BCL6	activation	BACH2	Alinikula Et Al., 2011	
BCL6	inhibition	BLIMP1	Martins2006, Johnston2010	Th1 th2 th17 cytokines
BCL6	inhibition	GATA3	Yu2009a, Nurieva2009, Ku	Postranscriptional
BCL6	inhibition	IFNG	Yu2009	
BCL6	activation	IL21	Yu2009, Zhu2010	
BCL6	inhibition	IL2RA	Johnston2012	
BCL6	inhibition	RORGT	Yu2009a, Nurieva2009	
BCL6	inhibition	TBET	Yu2009a, Nurieva2009	
BLIMP1	inhibition	BCL6	Martins2006, Johnston2009, Cimmino2008, Klein2003	
BLIMP1	activation	IRF4	DeSilva2012, Kallies2004	
BLIMP1	inhibition	PAX5	Kikuchi Et Al., 2012, Lin Et Al., 2002	
C-MAF	activation	IL10	Saraiva2009, Pot2009, Xu2	Direct
C-MAF	increase	IL4	Li1999	Cooperation junb, c-maf
C-REL	increase	FOXP3	Isomura2009, Ruan2009	
C-REL	increase	RORGT	Chen2011b	Cooperation c-rel, rorgt
C/EBP	activation	IL10	Ouyang2011	
CBFB	increase	FOXP3	Kitoh2009, Bruno2009, Rudra2009	
CMAF	activation	CD25	Hwang2002an	
CREB	activation	FOXP3	Leonard2007	Demethylation
CREB	activation	IL10	Ouyang2011	
DEC2	activation	GATA3	Veldhoen2008aryl	
DEC2	activation	IL2RA	Yang2009requirement Liu2009dec2	
EOMES	activation	GZMB	Intlekofer Et Al., 2005, Pearce Et Al., 2003	
EOMES	activation	IFNG	Intlekofer2008anomalous	Synergy tbet
EOMES	colaboration a	RUNX3	Zhu2010:Fig2	Th1
ETS1	activation	FOXP3	Mouly2010	Demethylation
ETS1	collaboration	TBET	Grenningloh2005ets-1, Mois	Th17
EZH2	inhibition	EOMES	Wang2013	
FAS	decrease	IFNGR1	Yosef2013	Prediction
FLT3L	activation	FLT3	Gwin Et Al., 2013	
FOXO1	activation	FOXP3	Ouyang2010	
FOXP3	inhibition	AKT	Roffe2010, Zhang2013	
FOXP3	decrease	CD28	Delacher2014	
FOXP3	activition	CTLA4	Katoh2011	
FOXP3	activation	FOXP3	Gavin2007	
FOXP3	activation	FR4	Kunisawa2012, Yamaguchi2007	
FOXP3	activation	GATA3	Rudra2012	Low
FOXP3	inhibition	GATA3	Zeng2009, Wan&Flavell2007, Williams&Rudensky2007	
FOXP3	increase	ICOS	Camperio2012	Foxp3::irf4 collaboration
FOXP3	inhibition	IFNG	Pan2009	Collaboration foxp3::eos::ctb
FOXP3	inhibition	IL2	Ono2007, Wu2006, Li2007,	Foxp3::nfat inhibits binding n
FOXP3	inhibition	IL6	Chaudhry2009	Foxp3::stat3-p binds il6 prom

CD4+ T cell interactions

FOXP3	complex	NFAT	Wu2006	
FOXP3	inhibition	NFAT	Bettelli2005foxp3	
FOXP3	inhibition	NFKB	Bettelli2005foxp3	
FOXP3	inhibition	PLU1/MOF	Katoh2011	
FOXP3	inhibition	RORGT	Zhou2008, Lochner2008, Ic	Foxp3::rorgt binding, inhibits
FOXP3	inhibition	TBET	Veldhoen2006, Zhong2007	
FOXP3	inhibition	TCR	Delacher2014	Signaling
FOXP3	activation	TGFB	Zhu2010	
FOXP3	activation	FOXP3	Pyzik07, Floess2007, Yao2007, Tone2008	
FOXP3	activation	NFAT	Pyzik07, Floess2007, Yao2007, Tone2008	
FOXP3	activation	RORGT	Pyzik07, Floess2007, Yao2007, Tone2008	
FOXP3	activation	STAT1	Pyzik07, Floess2007, Yao2007, Tone2008	
FOXP3	activation	STAT3	Pyzik07, Floess2007, Yao2007, Tone2008	
GATA3	complex	AP-1	Wei2011	
GATA3	activation	CD25	Zhu2010differentiation	
GATA3	activation	DEC2	Yang2009, Liu2009	
GATA3	complex	FLI1	Wei2011	Ets family member
GATA3	activation	FOXP3	Wohlfert2011, Wang2011	Low
GATA3	complex	FOXP3	Rudra2012	
GATA3	inhibition	FOXP3	Mantel2007, Wan&Flavell2007	
GATA3	activation	GATA3	Ouyang2000, Ranganath&Murphy2001	
GATA3	colaboration	GFI1	Zhu2010:Fig2	Th2
GATA3	inhibition	IFNG	Chang&Aune2007, Yagi201(	Gata3::runx3 sequesters run
GATA3	activation	IL10	Schmidt-Weber1999	
GATA3	activation	IL13	Zhu2004conditional Siegel1995activation Kishikawa2001(	
GATA3	activation	IL17	Zhu2010:Fig2	Th2
GATA3	activation	IL21	Zhu2010:Fig2	Low
GATA3	activation	IL4	Ouyang2000	Activation
GATA3	activation	IL4	Agarwal2000, Cote-Sierra2	Only binds enhancers, needs
GATA3	activation	IL9	Dardalhon2008	Indirect? Maybe stat6
GATA3	inhibition	RORGT	Park2005	
GATA3	inhibition	RORGT	Wohlfert2011	Binds rorc
GATA3	complex	RUNX3	Wei2011, Yagi2010, Kohu2009	
GATA3	inhibition	RUNX3	Zhu2010differentiation	Protein-protein iteraction
GATA3	inhibition	STAT-4	Usui Et Al., 2003	
GATA3	inhibition	STAT4	Usui2003, Vanhamburg2008	Decrease expression protein
GATA3	colaboration	STAT5	Zhu2010:Fig2	Th2
GATA3	inhibition	TBET	Zhu2008, Usui200, Wohlfert	Protein-protein iteraction
GATA3	activation	GATA3	Mendoza06	
GFI-1	colaboration	GATA3	Zhu2010:Fig2	Th2
GFI1	inhibition	SMAD3	Zhu2010differentiation	Actully tgfb functons are sup
HEB	inhibition	GATA3	Braunstein And Anderson, 2011	
HIF1-A	inhibition	FOXP3	Dang2011	Foxp3 ubiquitination
HLX	collaboration	TBET	Mullen2002hlx	Increase ifng
HOXA9	activation	FLT3	Gwin Et Al., 2010	
IFNG	activation	BCL6	Choi2013	
IFNG	activation	IFNGR	Horvath2004jak, Krause2006modulation	
IFNG	activation	IL10	Saraiva2009	
IFNG	activation	STAT1	Lighvani2001t-Bet Afkarian2002t-Bet Lieberman2004stat1	
IFNG	activation	TBET	Lighvani2001	
IFNG	activation	FOXP3	Djuretic2007, Bettelli2005, Mendoza06	

CD4+ T cell interactions

IFNG	activation	RUNX3	Djuretic2007, Bettelli2005, Mendoza06	
IFNG	activation	STAT4	Djuretic2007, Bettelli2005, Mendoza06	
IFNG	activation	TBET	Djuretic2007, Bettelli2005, Mendoza06	
IKAROS	activation	GFI1	Moroy&Khandanpour2011, Spooner2009	
IKAROS	inhibition	IFNG	Quirion2009cutting	
IKAROS	inhibition	PU.1	Predicted In This Work	
IKAROS	inhibition	TBET	Quirion2009cutting	
IKB\SIGMA	increase	IL17F	Okamoto2010	Ikb family member, cooperat
IKKB	activation	FOXP3	Schmidt-Supprian2003	
IL10	inhibition	IFNG	Eyles2002,Fujimoto2002	
IL10	activation	IL10	Rutz2011	
IL10	inhibition	IL2	Zhan2009,Lu2011	
IL10	inhibition	IL21	Taleb2009,Chen2006,Tanaka2008	
IL10	activation	IL9	Monteyne, Houssian1995	Synergy, non-direct, requiere
IL10	activation	SOCS2	Yoshimura2012	
IL10	activation	SOCS3	Yoshimura2012	
IL10	activation	STAT3	Ouyang2011	
IL10	activation	STAT5	Ouyang2011	Low
IL10	inhibition	TNFA	Han2014, Hotamisligil1999	
IL10	activation	GATA3	Naldi2010	
IL10	activation	NFAT	Naldi2010	
IL10	activation	PROLIFERAT	Naldi2010	
IL10	activation	STAT3	Naldi2010	
IL12	activation	TBX21	Hoey2003	Gene tbet
IL17	activation	FOXP3	Zhou2008	
IL2	activation	FOXP3	Villarino2007	
IL2	activation	GATA3	Zhu2010	
IL2	activation	IL10	Houssian1995	Synergy
IL2	activation	IL2R	Cote2004interleukin, Zhu2003stat5,	
IL2	activation	STAT5	Kagami2001	
IL2	activation	FOXP3	Villarino2007,	
IL2	activation	STAT5	Villarino2007,	
IL2	activation	TBET	Villarino2007,	Low
IL21	activation	BCL6	Nurieva2009	
IL21	inhibition	EOMES	Suto2006il-21	May also be expressed as il2
IL21	inhibition	FOXP3	Korn2007	
IL21	inhibition	GATA3	Yang2007	
IL21	activation	IL10	Pot2009, Saraiva2009	Synergy
IL21	activation	IL21	Zhou2007, Nurieva2007	Requires stat3 and rorgt
IL21	activation	IL23R	Zhou2007	
IL21	activation	RORGT	Goreschi2010, Yang2007, Z	Synergy
IL21	activation	STAT3	Veldhoen2006tgfβ Zhou2007il-6 Nurieva2007essential Kc	
IL21	activation	STAT3	Mcgeachy07, Mendoza06a	
IL22	activation	STAT5	Ouyang2011	Low
IL23	inhibition	IL10	Mcgeachy2007	
IL23	activation	RORGT	Goreschi2010, Yang2007, Z	Collaboration il6, il23 & il1b,
IL23	activation	TBET	Goreschi2010	Low
IL23R	activation	STAT3	Mcgeachy2009interleukin	
IL23R	activation	STAT3	Ivanov07	
IL27	activation	IL10	Stumhofer2007, Pot2009	Synergy tgfb
IL27	activation	TGFB	Stumhofer2007, Pot2009	Synergy il27

CD4+ T cell interactions

IL2R	activation	MTOR	Stahl2002	
IL2R	activation	IL2	Kim2006	
IL2R	activation	IL2RB	Kim2006	
IL2RA	activation	FOXP3	Kim06	
IL2RA	activation	NFAT	Kim06	
IL2RA	activation	NFKB	Kim06	
IL2RA	activation	SMAD3	Kim06	
IL4	activation	GATA3	Murphy2002	
IL4	activation	GFI1	Zhu2002growth	
IL4	activation	IL10	Houssian1995	Synergy
IL4	activation	IL4R	Chen2003	
IL4	activation	PPARG	Cunard2002regulation	
IL4	activation	FOXP3	Djuretic2007, Bettelli2005, Elser02	
IL4	activation	GATA3	Djuretic2007, Bettelli2005, Elser02	
IL4	activation	TBET	Djuretic2007, Bettelli2005, Elser02	
IL6	activation	IL10	Mcgeachy2007	Synergy
IL6	activation	IL21	Zhou2007, Wei2007, Korn20	Requires only stat3
IL6	inhibition	IL2RA	Choi2013	
IL9	activation	SOCS3	Lejuen2001	Low
IL9	activation	STAT5	Homakova2009	Homo y hete
IRAK1	activation	IFNG	Okamura1998regulation	Synergy il12/stat4
IRF1	activation	IL10	Ouyang2011	
IRF4	increase	BCL6	Kwon2009	Collaboration stat3
IRF4	activation	BLIMP1	Nutt Et Al., 2011, Sciammas Et Al., 2006	
IRF4	complex	FOXP3	Zheng2009	Modulation various genes
IRF4	inhibition	FOXP3	Zheng2009	Requiered for rorgt inhibition
IRF4	increase	GATA3	Rengarajan2002a, Lohoff20	Normal il4 signaling
IRF4	increase	ICOS	Camperio2012	Foxp3::irf4 collaboration
IRF4	increase	IL10	Tian2011, Huber2011, Chaudhry2011, Ahyi2009	
IRF4	inhibition	PAX5	Decker Et Al., 2009, Nutt Et Al., 2011	
IRF4	increase	RORGT	Zheng2009, Brustle2007	
IRF4	complex	STAT3	Kwon2009	
JUNB	increase	IL4	Li1999	Cooperation junb, c-maf
MINA	decrease	FOXP3	Yosef2013	Prediction
MTOR	activation	FOXP3	Hedrick2012	
NFAT	activation	FOXP3	Delacher2014, Tone2008	Necessary, collaboraton sma
NFAT	decrease	IL10	Ranger1998b	Nfat1, nfatc2&nfatc3
NFKB	activation	FOXP3	Long2009, Ruan2009, Zheng2010	
NFKB	activation	IL10	Ouyang2011	
NFKB	increase	TBET	Corn2003	
NOTCH	activation	GATA3	Amsen2007direct, Fang2007	Synergy
NOTCH	activation	IL10	Ouyang2011	
Opera	activation	FOXP3	Uchiyama2012	Ignobel 2012
P50	increase	GATA3	Das2001	Requiered early induction
PAX5	activation	BACH2	Kallies And Nutt, 2010, Kikuchi Et Al., 2012	
PAX5	inhibition	BLIMP1	Yasuda Et Al., 2012	
PAX5	inhibition	FLT3	Holmes Et Al., 2006	
PKCO	increase	FOXP3	Gupta2008	
POU2AF1	decrease	FOXP3	Yosef2013	Prediction
POU2AF1	decrease	GATA3	Yosef2013	Prediction
PPARG	inhibition	AP1	Ricote1998peroxisome	Macrophages epithelial

PPARG	inhibition	IFNG	Cunard2004repression	
PPARG	inhibition	IL2	Chung2003inhibition	Synergy nfat
PPARG	inhibition	IL4	Yang2002interleukin	
PPARG	inhibition	IL6R	Ricote1998peroxisome	
PPARG	inhibition	NFAT	Chung2003inhibition	
PPARG	inhibition	NFKB	Ricote1998peroxisome	Macrophages epithelial
PPARG	inhibition	RORGT	Klotz2009nuclear	
PPARG	inhibition	STAT3	Wang2004transcriptional	
PU.1	inhibtion	IRF4	Ahyi2009	Irf4::pu.1 block binding to il4,
RELB	activation	TBET	Corn2005	Necessary
RORGT	complex	BATF	Ciofani2012	Modulate gene expression
RORGT	inhibition	FOXP3	Bettelli2006, Burgler2010	
RORGT	inhibition	GATA3	Bettelli2006, Veldhoen2006	
RORGT	activation	IL17	Yang2008t, Yang2007, Math	Requires stat3
RORGT	complex	IRF4	Ciofani2012	Modulate gene expression
RORGT	complex	RUNX1	Lazarevic2012	Necessary rorgt activity
RORGT	complex	STAT3	Ciofani2012	Modulate gene expression
RORGT	inhibition	TBET	Ivanov2006, Laurence2007	Inhibition
RORGT	activation	STAT3	Zhou07, Manel2008	Protein
RORGT	activation	TGFBR	Zhou07, Manel2008	
RUNX1	activation	EBF1	Seo Et Al., 2012	
RUNX1	activation	FOXP3	Klunker2009, Bruno2009, Ki	Runx1::nr4a2 collaboration,
RUNX1	inhibition	GATA3	Wong Et Al., 2011	
RUNX1	activation	IL17	Zhang2008interactions	
RUNX1	activation	IL2	Ono2007foxp3, Kitoh2009,	In th naive
RUNX1	inhibition	IL4	Naoe2007, Kitoh2009	
RUNX1	activation	RORGT	Zhang2008interactions	
RUNX1	complex	RORGT	Zhang2008	
RUNX3	inhibition	EBF1	Mendoza2014	
RUNX3	activation	EOMES	Cruz-Guilloty2009runx3	
RUNX3	activation	FOXP3	Klunker2009, Bruno2009, Kitoh2009, Rudra2009	
RUNX3	inhibition	GATA3	Yagi2010, Hwang2005b, Ko	Collaboration runx3::tbet to i
RUNX3	activation	IFNG	Djuretic2007, Kohu2009, Ya	Collaboration tbet, stat4
RUNX3	inhibition	RUNX1	Giambra2012	
RUNX3	colaboration	TBET	Zhu2010:Fig2	Th1
RUNX3	activation	TBET	Djuretic2007	
SMAD3	activation	FOXP3	Gu2012, Malhotra2010, Chen2003, Li2007, Marie2006, T	
SOCS1	inhibition	IFNGR	Yoshimura2012	
SOCS1	inhibition	IL2R	Yoshimura2012	
SOCS1	inhibition	IL4R	Yoshimura2012, Losman1999il4	
SOCS1	inhibition	STAT1	Cooney2002, Kimura2004, E	Il6r
SOCS2	inhibition	IL2R	Yoshimura2012	
SOCS3	inhibition	IFNGR	Yoshimura2012	
SOCS3	inhibition	IL23R	Yoshimura2012	
SOCS3	inhibition	IL6R	Yoshimura2012	
SOCS3	activation	IL9	Lejuen2001	
SOCS3	inhibition	IL9	Chen2006	
SOCS3	inhibition	STAT3	Taleb2009,Chen2006	Il6r, il23r
SOCS5	inhibition	IL4R	Yoshimura2012	
SP1	activation	IL10	Ouyang2011	
STAT1	activation	BCL6	Choi2013	

CD4+ T cell interactions

STAT1	activation	IL10	Saraiva2009	
STAT1	activation	SOCS1	Chen2000, Saito2000, Sato2004, Yoshimura2012	
STAT1	activation	SOCS2	Yoshimura2012	
STAT1	activation	SOCS3	Yoshimura2012, Choi2013	
STAT1	activation	TBET	Lighvani2001t-Bet Afkarian2002t-Bet Lieberman2004stat1	
STAT1	activation	IFNGR	Mendoza06, Weaver07, Kamiya2004	
STAT3	activation	BATF	Durant2010	
STAT3	activation	BCL6	Nurieva2009, Choi2013, Oes	Il6 e il21
STAT3	activation	FOXO1	Kousteni2012	
STAT3	inhibition	FOXP3	Korn2007, Yang2007, Xu20(	Mediated by competition with
STAT3	inhibition	GATA3	Yang2007stat3	
STAT3	activation	IL10	Saraiva2009, Tian2011, Huber2011, Chaudhry2011	
STAT3	activation	IL21	Wei2007, Zhu201, Durant20	Rorgt independent
STAT3	activation	IL23R	Zhou2007, Nurieva2007, Yang2007, Chung2003	
STAT3	activation	IRF4	Durant2010	
STAT3	activation	RORGT	Zhou2007 Nurieva2007, Yan	Optimal requieres tgfb
STAT3	activation	SOCS1	Yoshimura2012	
STAT3	activation	SOCS3	Choi2013	
STAT3	inhibition	TBET	Yang2007stat4	
STAT3	inhibition	TGFB	Chaudhry2009	Colaboration foxp3::stat3
STAT3	activation	IL10R	Brenne2002, Mendoza06a, Weaver07	
STAT3	activation	IL23R	Brenne2002, Mendoza06a, Weaver07	
STAT3	activation	IL6R	Brenne2002, Mendoza06a, Weaver07	
STAT4	activation	IFNG	Usui2006, Usui2003, Park2004	
STAT4	activation	IL10	Saraiva2009	
STAT4	activation	SOCS5	Yoshimura2012	
STAT4	activation	TBET	Usui2006t-Bet Usui2003gata-3	
STAT4	activation	GATA3	Mendoza06a	
STAT5	inhibition	BCL6	Oesterich2012, Scheeren2005, Cihangir2010	
STAT5	activation	FOXP3	Davidson2007cutting Burchil	Davidson2007, burchill2007,
STAT5	activation	GATA3	Guo2009	Only maintains
STAT5	activation	IL2RA	Johnston2012	
STAT5	activation	IL4	Zhu2003, Hural2000	Accesibility hsii site in il4 locu
STAT5	activation	PAX5	Hirokawa Et Al., 2003	
STAT5	inhibition	TBET	Zhu2010:Fig2	Low
STAT5	activation	IL2R:1	Kim06	
STAT5	activation	IL2R:2	Kim06	
STAT6	inhibition	FOXP3	Dardalhon2008il4	
STAT6	activation	GATA3	Kurata1999, Zhu2001, Chen2003, Onodera2010	
STAT6	activation	GFI1	Zhu2010:Fig2	Th2
STAT6	inhibition	IFNG	Chang&Aune2007, Szabo20(	Collaboration gata3, chroma
STAT6	activation	SOCS3	Yoshimura2012	
TAK1	activation	FOXP3	Wan2006, Sato2006	
TBET	inhibition	BCL6	Kenneth2011, Oesterich201	Tbet::bcl6 inhibits bcl6-media
TBET	inhibition	C-REL	Hwang2005	Tbet::c-rel inhibits c-rel activi
TBET	activation	EOMES	Morishima Et Al., 2005	
TBET	inhibition	FOXP3	Veldhoen2006, Zhong2007	
TBET	inhibition	GATA3	Usui2006, Hwang2005, Szal	Collaboration runx3::tbet to i
TBET	activation	IFN-G	Szabo Et Al. (2000)	Activation
TBET	activation	IL12RB2	Mullen2001role Mullen2002l	Synergy eomes
TBET	inhibition	IL23R	Mathur2006Tbet, Gocke2007	Tbet

CD4+ T cell interactions

TBET	inhibition	IL4	Zhu2010differentiation	
TBET	colaboration a	RUNX3	Zhu2010:Fig2	Th1
TBET	activation	SOCS1	Szabo2000, Losman1999il4	
TBET	inhibition	RORGT	Lazarevic2011, Villarino201	Sequester runx1, runx3
TBET	complex	RUNX1	Lazarevic2012	Inhibit rorgt activity
TBET	complex	RUNX3	Lazarevic2013, Yagi2010,	Inhibit gata3, rorgt
TBET	inhibition	SOCS1	Oesterich2011, Oesterich20	Bcl6::tbet complex
TBET	inhibition	SOCS3	Oesterich2011, Oesterich20	Bcl6::tbet complex
TBET	activation	TBET	Kanhere2012, Mullen2001, A	Cooperation stat1, maintain
TBET	activation	TBET	Mendoza06	
TBET	inhibition	TCF7	Oesterich2011, Oesterich20	Bcl6::tbet complex
TBET				
TCF-1	activation	GATA3	Rothenberg, 2012	
TCR	inhibition	RUNX1	Wong Et Al., 2011	
TGFB	activation	FOXP3	Korn2007, Zhou2008, Chen2	Suppresed by stat3
TGFB	inhibition	GATA3	Gorelik2000	
TGFB	inhibition	GFI1	Zhu2010differentiation	
TGFB	activation	IL10	Mcgeachy2007, Zheng2007,	Synergy
TGFB	inhibition	IL23R	Lee2012b	
TGFB	activation	SMAD3	Chen2003conversion Li2007	+ tcr
TGFB	activation	TIEG1	Venuprasad2008e3	
TIEG1	activation	FOXP3	Venuprasad2008, Cao2009	Induced by tgfb coperation e
TSC22D3	increase	FOXP3	Yosef2013	Prediction

Node	Function
T-bet	(Th1_s or T-bet) and not GATA3
GATA3	(Th2_s or GATA3) and not T-bet
Foxp3	(iTreg_s or Foxp3) and not RORγt
RORγt	Th17_s and not (T-bet or GATA3 or Foxp3)
Bcl6	Tfh_s and not T-bet

Node	Function
TBET	(IFNG or TBET) and not (IL4 or GATA3 or IL21 or BCL6 or IL9)
IFNG	(IFNGe or ((IFNG or TBET) andnot (GATA3 or TGFB or BCL6))) and not (IL21 or IL4 or IL10 or IL9)
GATA3	((IL2 and IL4) or GATA3) and not (TBET or IFNG or TGFB or IL21 or BCL6)
IL2	(IL2e or (IL2 and not FOXP3)) and not (IFNG or IL21 or IL10)
IL4	(IL4e or (GATA3 and (IL2 or IL4) and not TBET)) and not (IFNG or IL21)
RORGT	(IL21 and TGFB) and not (TBET or FOXP3 or GATA3 or BCL6)
IL21	(IL21e or IL21 or RORGT or BCL6) and not (IFNG or IL4 or IL10 or IL2 or IL9)
FOXP3	(IL2 and (TGFB or FOXP3)) and not (IL21 or RORGT)
TGFB	TGFBe or ((TGFB or FOXP3) and not IL21)
IL10	IL10e or (IL10 and (IFNG or IL21 or TGFB or GATA3))
BCL6	(IL21) and not (TBET or IL2 or TGFB)
IL9	(IL4 and ((IL10 and IL2) or TGFB)) and not (IFNG or IL21)

Supplementary Material
Network Simplification

Mariana Martinez-Sanchez Luis Mendoza Carlos Villarreal
Elena R. Alvarez-Buylla

The differentiation and plasticity of CD4+ T lymphocytes is the result of the concerted action of many components like cytokines, receptors, transcription factors, etc. The large number of components makes it convenient to simplify the resulting network.

1 Construction of the logical functions

We considered that a node is active if there is enough amount of protein or gene expression to be functional and affect the differentiation of CD4+ T lymphocytes. A transcription factor is active if it is present in enough quantity and in a conformation that can alter the expression of its target genes. A transcription factor or cytokine is active if it is present in enough quantity and in a conformation that can form a functional complex with its receptor. A receptor is active if it forms a complex that can activate its downstream signaling. A STAT proteins is active if it is phosphorylated and forms a dimer capable of translocating to the nucleus and affecting the expression of its target genes.

Basal levels

A protein or gene may be expressed at a basal level, but does not necessarily affect the differentiation of the cell at that level of expression. For example, GATA3 is necessary for T cell maduration and for CD4+ T-cell survival and maintenance. The deleterious mutation of GATA3 is letal, and Lck-Cre conditional deletion models lack CD4+ T cells or have impaired survival and maintainance. GATA3^{high} also drives the differentation into Th2 (Ho, Tai and Pai 2009). In this case we considered that the basal level of GATA3^{low} corresponded to zero, while GATA3^{high} was one.

Expression	Phenotype	Node value
GATA3^{KO}	Letal	-
GATA3^{low}	Survival	0
GATA3^{high}	Th2	1

Weak interactions

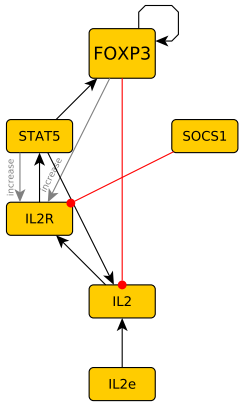
Weak interactions were ignored in our model. Interactions between genes and proteins are weak when they increase or decrease the expression of a gene or protein but are not necessary or sufficient to cause changes in differentiation. For example, The IL-2 receptor (IL2-R) is necessary for the activation of CD4+ T cells and plays a central tole in the differentiation towards Th2 and iTreg. IL-2R is composed of three subunits IL-2Rα, IL-2Rβ, γ_c. The three subunits together form a high affinity receptor, while IL-2Rβ and γ_c form a medium affinity receptor, both complexes are

functional. IL2 increases the expression of IL-2Rα and IL-2Rβ and Foxp3 increases the expression of IL-2Rα. The result is that the IL-2R can form a functional complex (IL2R = 1) in the presence of IL-2 with or without Foxp3, even if the transcription factor affects its expression levels and affinity (Liao 2011).

IL-2$_t$	Foxp3$_t$	IL2-R$_{t+1}$
0	0	0
0	1	0
1	0	1
1	1	1

2 Boolean Logic Reduction Method

To simplify the network we employed a Boolean reduction method proposed in Villarreal *et al*, 2012. For simplicity, we illustrate only the simplification scheme of the interactions between IL-2 and Foxp3. Interleukin 2 (IL-2) can be produced by the T CD4+ lymphocytes or by other cells of the immune system (IL2e). IL-2 binds the IL-2 receptor (IL-2R), which causes the phosphorylation and dimerization of STAT5. The phosphorylation of STAT5 can be inhibited by SOCS1, which binds the IL-2R. STAT5 activates the transcription of IL-2, Foxp3 and increases the transcription of IL-2R. Foxp3 can induce its own transcription and inhibit the transcription of IL-2. These interactions can be characterized by a set of logical propositions which satisfy the following mapping:



$$
\begin{aligned}
IL2_{t+1} &= IL2e_t \text{ or } (STAT5_t \text{ and not } FOXP3_t) \\
IL2R_{t+1} &= IL2_t \text{ and not } SOCS1_t \\
STAT5_{t+1} &= IL2R_t \\
FOXP3_{t+1} &= STAT5_t \text{ and } FOXP3_t
\end{aligned}
\tag{1}
$$

Considering that the expression level of node N at a time t is represented by N_t the attractors (steady states) that represent different phenotypes are determined by the condition $N_{t+1} = N_t$. In that case, the mapping becomes a set of coupled Boolean algebraic equations. The explicit expressions of the attractors are then obtained by performing the algebraic operations according to the axiomatic of Boolean algebra (see Villarreal *et al*, 2012):

$$
\begin{aligned}
IL2 &= IL2e \text{ or } (STAT5 \text{ and not } FOXP3) \\
IL2R &= IL2 \text{ and not } SOCS1 \\
STAT5 &= IL2R \\
FOXP3 &= STAT5 \text{ and } FOXP3
\end{aligned}
\tag{2}
$$

This results in the identity:

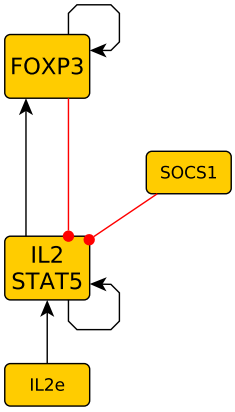
$$STAT5 = IL2R \tag{3}$$

We employ this identity to determine the system's attractors:

$$STAT5 = IL2 \text{ and not } SOCS1 \tag{4}$$

$$STAT5 = (IL2e \text{ or } (STAT5 \text{ and not } FOXP3)) \text{ and not } SOCS1 \tag{5}$$

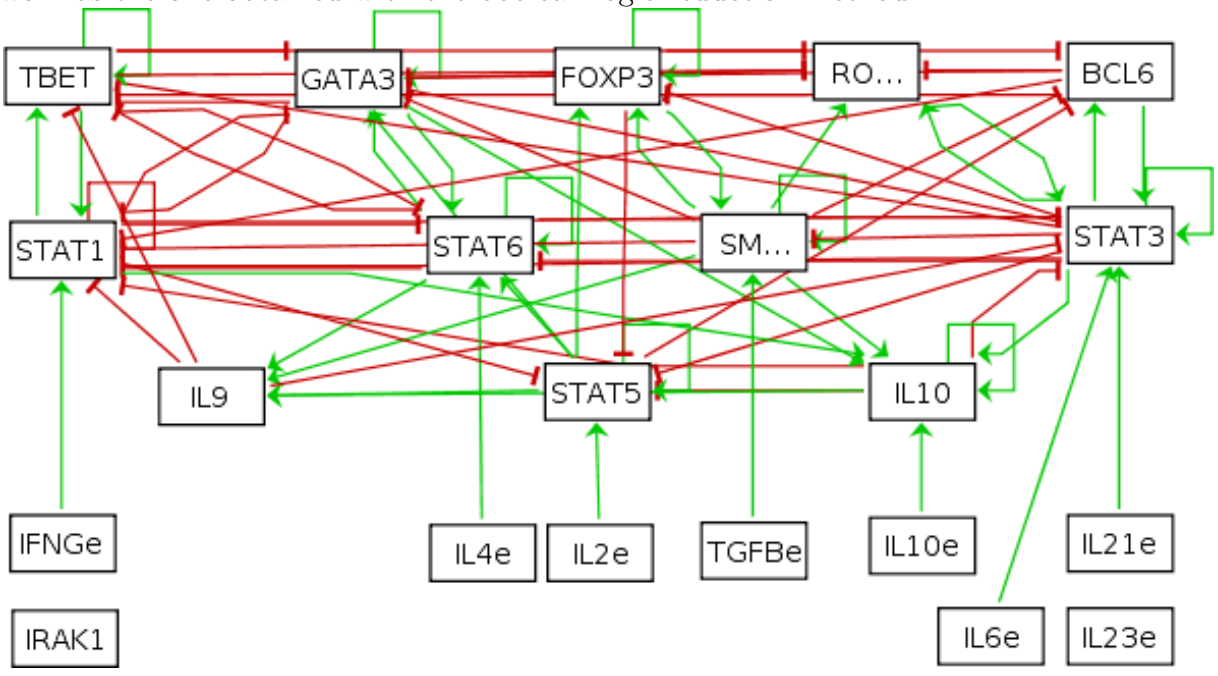
Thus, the regulatory network attractors are summarized by the expression values of the nodes pertaining to a concise set of Boolean expressions:



$$\begin{aligned} STAT5 &= (IL2e \text{ or } (STAT5 \text{ and not } FOXP3)) \text{ and not } SOCS1 \\ FOXP3 &= STAT5 \text{ and } FOXP3 \end{aligned} \tag{6}$$

3 Reduction of logical regulatory graphs

To verify the Boolean approach we compared our results with those obtained with the software GINsim (Naldi *et al*, 2009). GINsim uses decision diagrams to iteratively remove regulatory components and actualizes the components to maintain the indirect effects. The method preserves the dynamical properties of the original model. The simplification with GINsim returns a similar network as the one obtained with the boolean logic reduction method.



The simplification with GINsim recovers the same attractors as the Boolean logic reduction method, except for RORγt+STAT1+, Bcl6+STAT1+, and Foxp3+IL4+IL9+ attractors. The RORγt+STAT1+ and Bcl6+STAT1+ attractors arise from the codification of inhibitory interactions; our model assumes that inhibitions are strong, while GINsim assumes that enough activators may overcome an inhibition. The Foxp3+IL4+IL9+ attractor requires both IL2e and IL4e; under transient perturbations it transits towards Th9 (IL10+, IL2e-, IL10e+), iTreg (IL4e+), Th1R (IFNGe-) or Tfh (Bcl6+, IL21e+).

Name	TBET	GATA3	RORGT	FCXP3	BCL6	STAT1	STAT5	STAT6	STAT3	SMAD3	IL10	IL9	IRAK1	IFNGe	IL2e	IL4e	IL6e	IL21e	IL23e	TCFBe	IL10e
													*						*		*
										1			*						*	*	
										1	1		*		*			1	*	*	*
										1	1		*		*		1	*	*	*	*
								1					*			1	*		*		*
								1		1		1	*			1	*		*	*	
								1		1	1	1	*		*	1	*	*	*	*	*
							1						*		*				*		*
					1				1				*		*	*	*	*	*		
					1	1			1				*	1	*	*	*	*	*		
				1			1			1			*		1				*	*	
				1			1	1		1		1	*		1	1	*		*	*	
			1						1	1			*		*	*	*	*	*	1	
			1			1			1	1			*	1	*	*	*	*	*	1	
		1											*						*		
		1									1		*		*			1	*		*
		1									1		*		*		1	*	*		*
		1						1					*			*	*		*		
		1						1			1		*		*	*	*	*	*		*
		1					1	1					*		*	*	*		*		
	1									1			*						*	*	
	1									1	1		*		*			1	*	*	*
	1									1	1		*		*		1	*	*	*	*
	1					1							*	1	*	*	*		*		
	1					1					1		*	1	*	*	*		*		*
	1					1				1			*	1	*	*	*		*	*	
	1					1				1	1		*	1	*	*	*		*	*	*
	1			1			1			1			*		1				*	*	

Supplementary Material
Network Simplification

Mariana Martinez-Sanchez Luis Mendoza Carlos Villarreal
Elena R. Alvarez-Buylla

Boolean GRNs are useful to study the complex logic of transcriptional regulation involved in cell differentiation. However, a comprehensive understanding of the mechanisms participating in cell fate dynamics must take into account the continuos character of the variables involved in the description: levels of genetic expression, differences in concentrations, in decay rates, threshold expression values, etc. These factors may be taken into account by translating the discrete dynamical mappings describing GRN interactions into a set of differential equations. In order to get formal consistency of both descriptions, the new variables and operators that constitute the logical propositions must satisfy a generalization of Boolean axiomatics into the continuous realm. For that purpose, we transform the logical connectors *and*, *or*, and *not* according to the following operations:

$$a \text{ and } b \to a \cdot b \qquad a \text{ or } b \to a + b - a \cdot b \qquad \text{not } b \to 1 - b. \tag{1}$$

It is straightforward to show that these rules satisfy the axioms of Boolean algebra. We may then transform the Boolean logical propositions by direct substitution. An example is given by:

$$(a \text{ or } b) \text{ and not } c \to [a + b - a \cdot b]\ (1 - c).$$

We now consider the following set of differential equations defined by step-like inputs $\Theta[w_i]$, where w_i is a continuous logical proposition:

$$\frac{dx_i}{dt} = \Theta\left[w_i(x_1, ..., x_n) - \theta_i\right] - \alpha_i x_i. \tag{2}$$

Here, θ_i is a threshold value (usually, $\theta_i = 1/2$), while $\Theta\left[w_i(x_i - \theta_i)\right]$ is a logistic functional whose value is 1 if $w_i > \theta_i$, 1/2 if $w_i = \theta_i$, and 0 if $w_i < \theta_i$. α_i represents the decay rate for the expression of node i. A representation of $\Theta[w_i]$ is

$$\Theta[w_i] = \frac{1}{\exp\left[-\beta(w_i - \theta_i)\right] + 1}, \tag{3}$$

where β is a saturation rate. For $\beta \gg 1$, the functional $\Theta[w_i]$ becomes a Heaviside step function: $\Theta[w_i - \theta_i] \to H[w_i - \theta_i]$.

It may be shown that when all $\alpha_i = 1$, the steady states of the set (2), defined by $dx_i/dt = 0$, coincide with attractor set provided by the discrete Boolean approach, indicating the robustness of the continuous analysis.

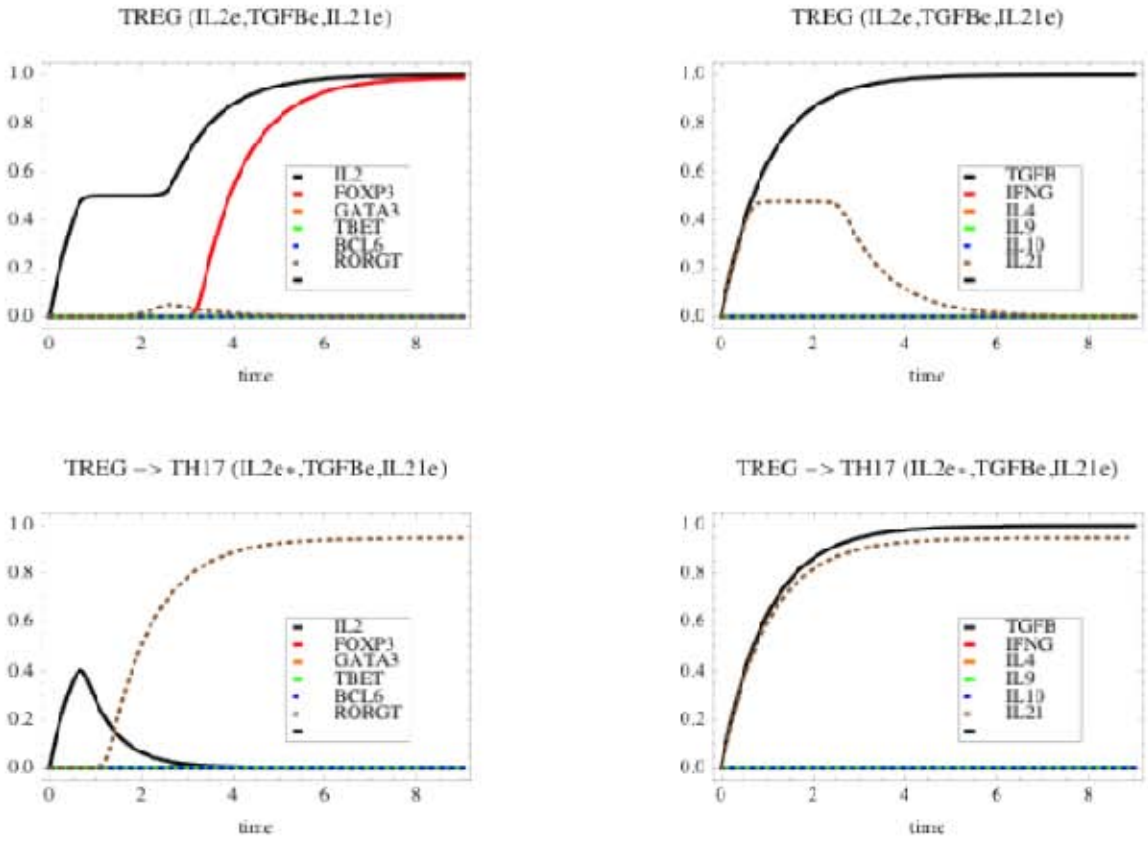
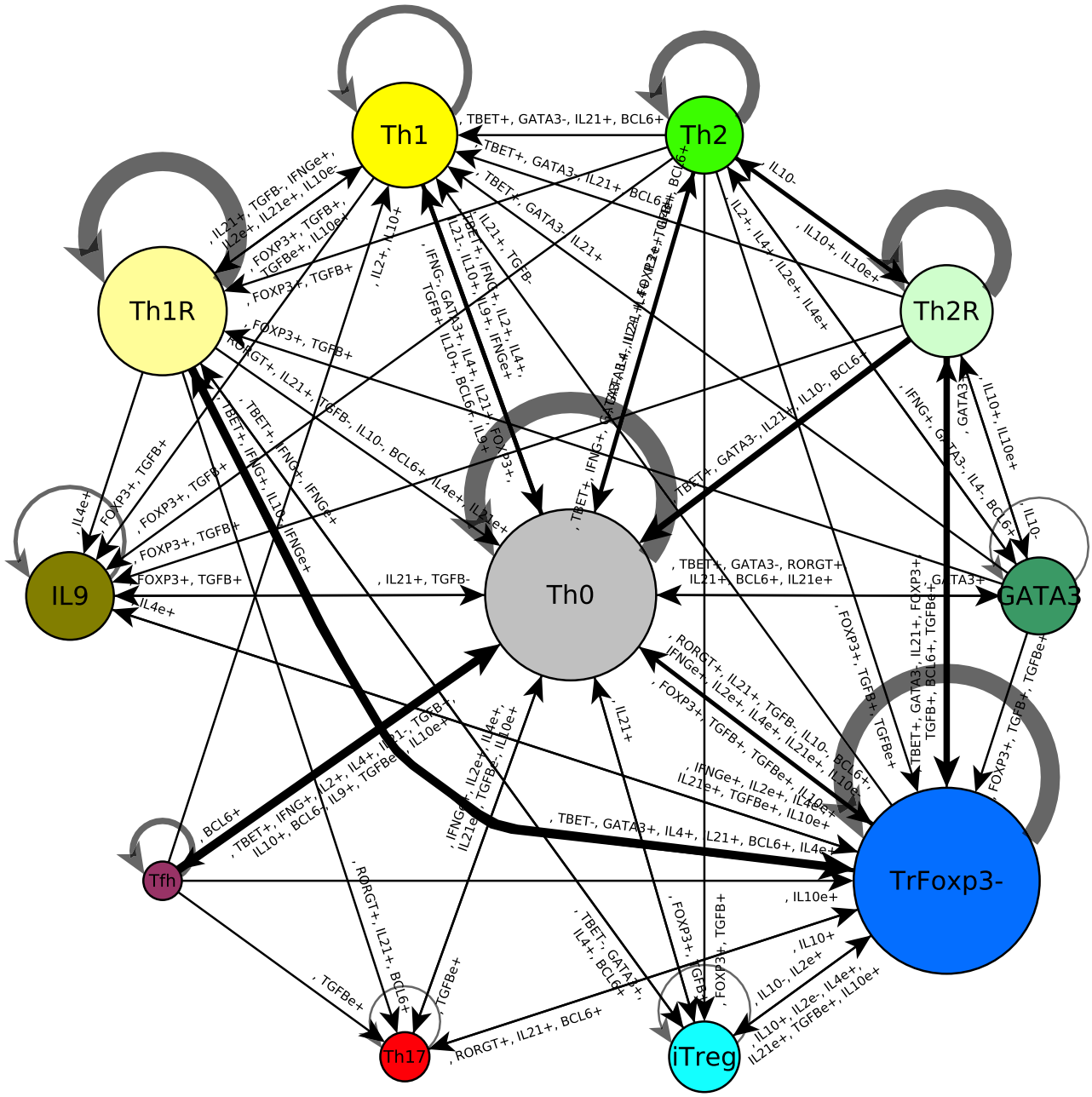
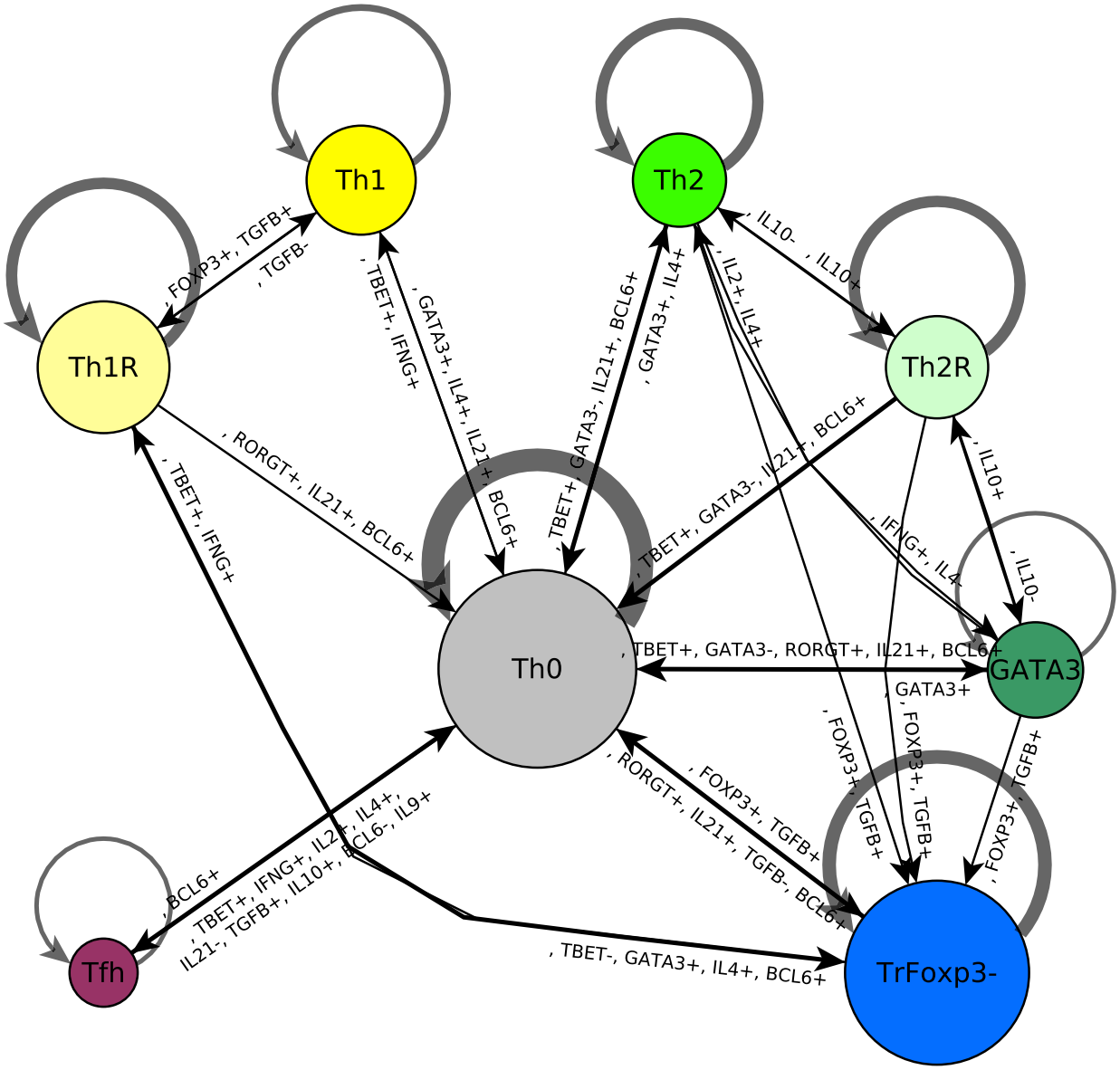


Figure 1: Differentiation and plasticity of CD4+ T cells in the continuous model. (A & B) Differentitation of Treg cells in response to IL2e, TGFBe and IL21e in the micro-environment. (C & D) Transition from Treg to Th17 in response to perturbations in IL2e.

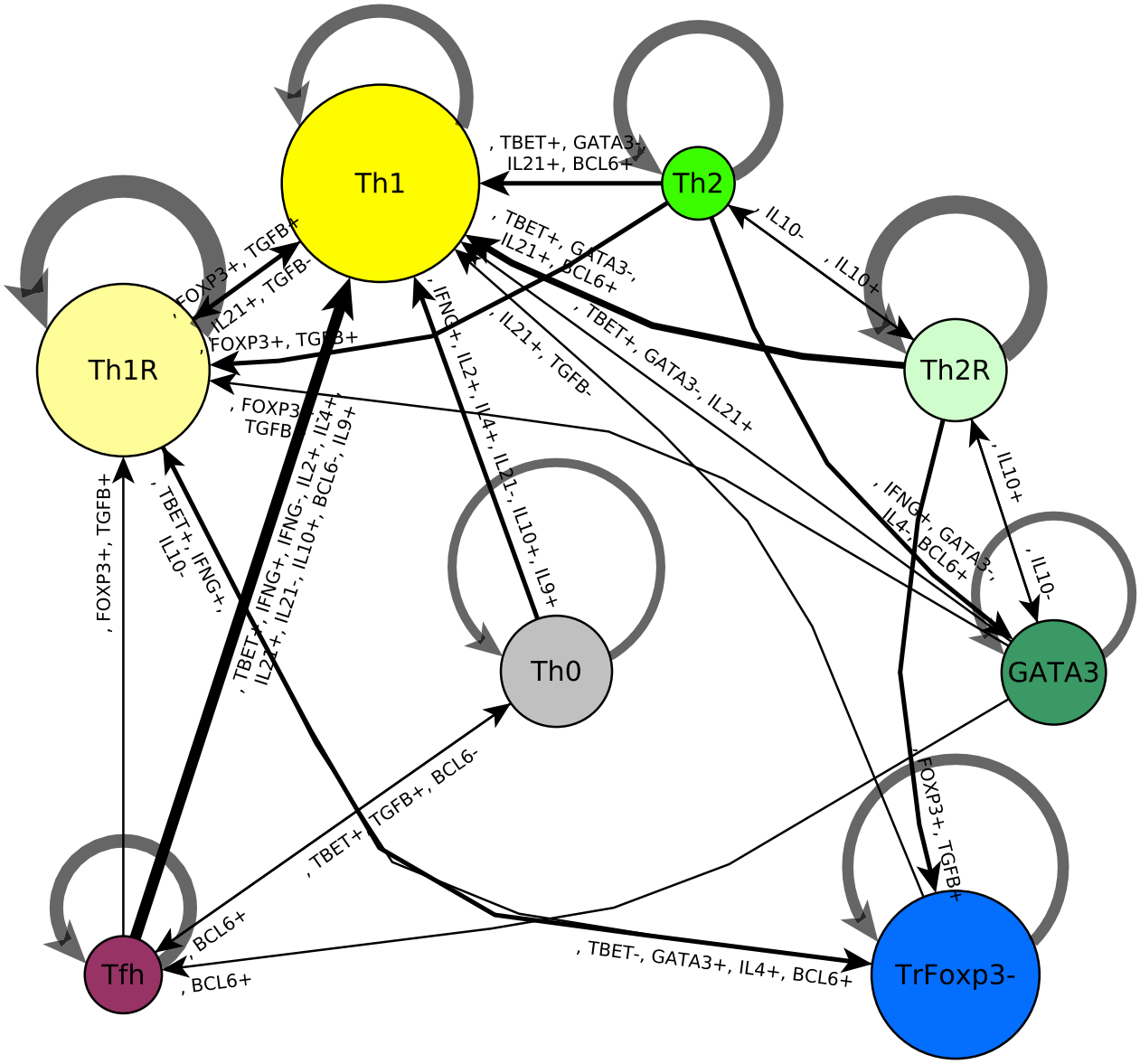
Transitions in response to transient perturbations in the nodes of the T CD4+ minimal regulatory network



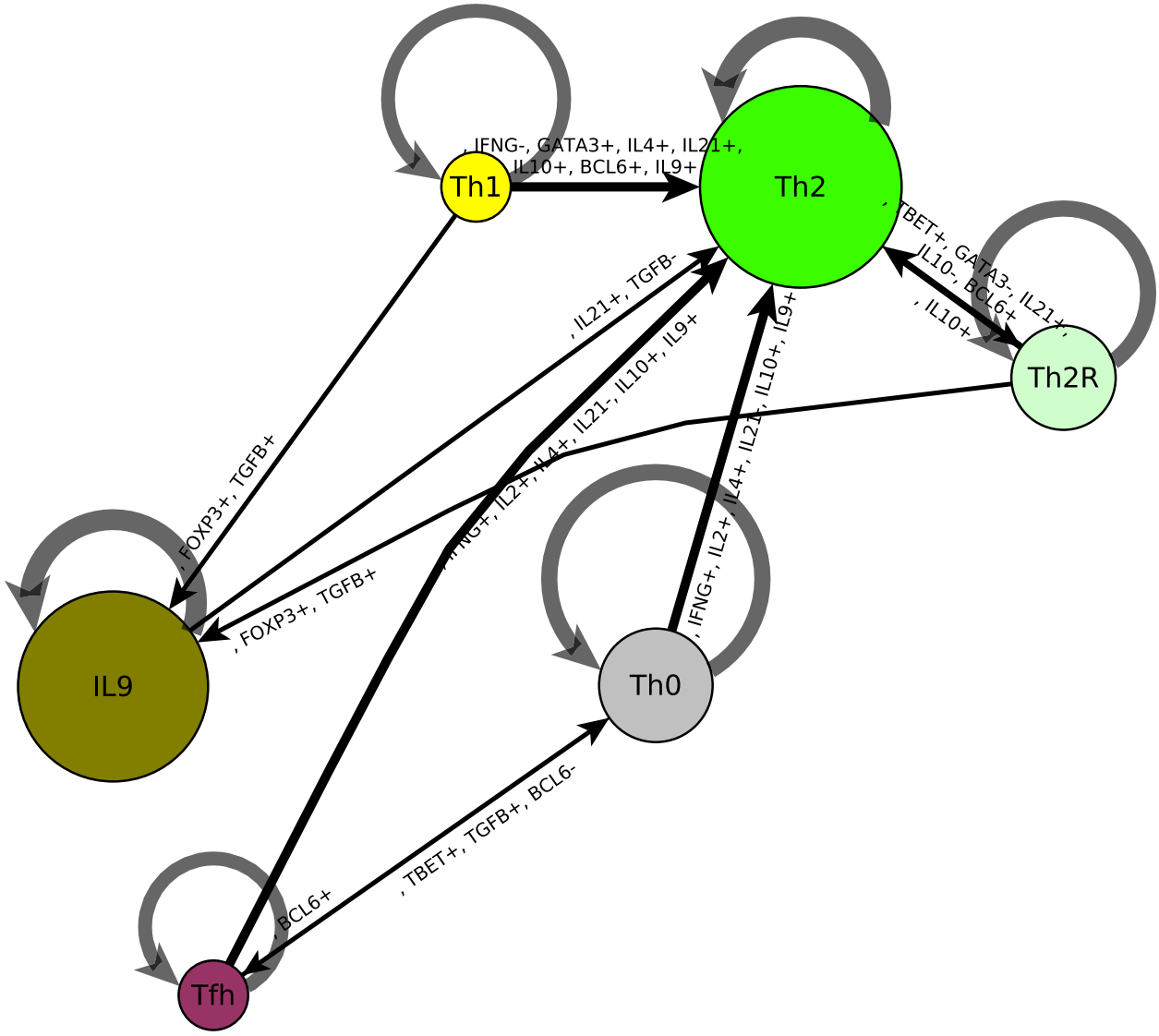
Th0 (No extrinsic signals)



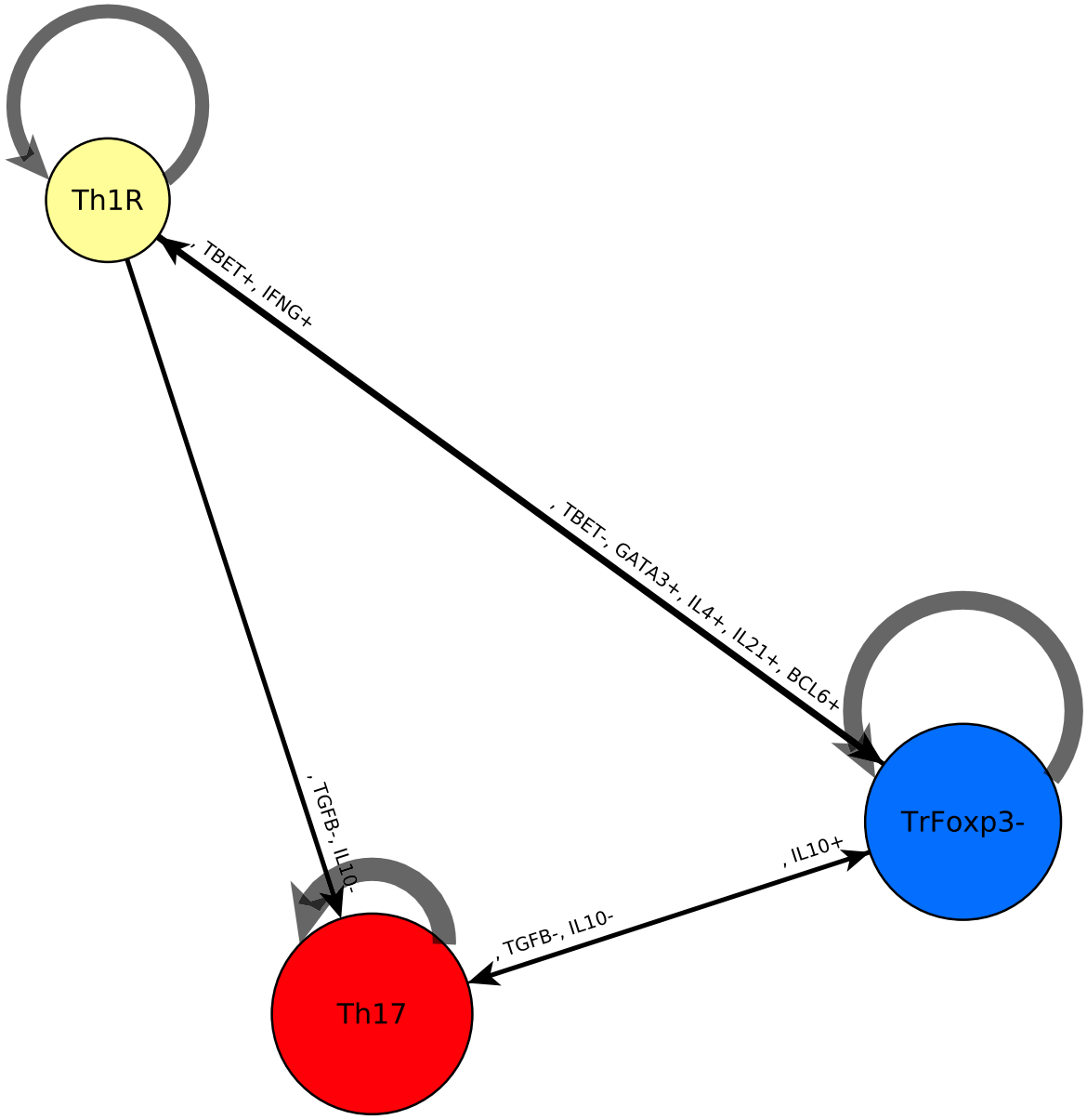
Th1 (IFNγe+)


Th1
Th2
Th1R
Th2R
Th0
GATA3
Tfh
TrFoxp3-
, TBET+, GATA3-, IL21+, BCL6+
, IL10-
, IL10+
, FOXP3+, TGFB+
, IL21+, TGFB-
, FOXP3+, TGFB+
, IFNG+, IL2+, IL4+, IL21-, IL10+, IL9+
, IL21+, TGFB-
, TBET+, GATA3-, IL21+
, FOXP3+, TGFB+
, IFNG+, GATA3-, IL4-, BCL6+
, IL10+
, IL10-
, TBET+, IFNG+, IFNG-, IL2+, IL4+, IL21-, IL10+, BCL6-, IL9+
, FOXP3+, TGFB+
, TBET+, IFNG+, IL10-
, TBET+, TGFB+, BCL6-
, FOXP3+, TGFB+
, BCL6+
, BCL6+
, TBET-, GATA3+, IL4+, BCL6+

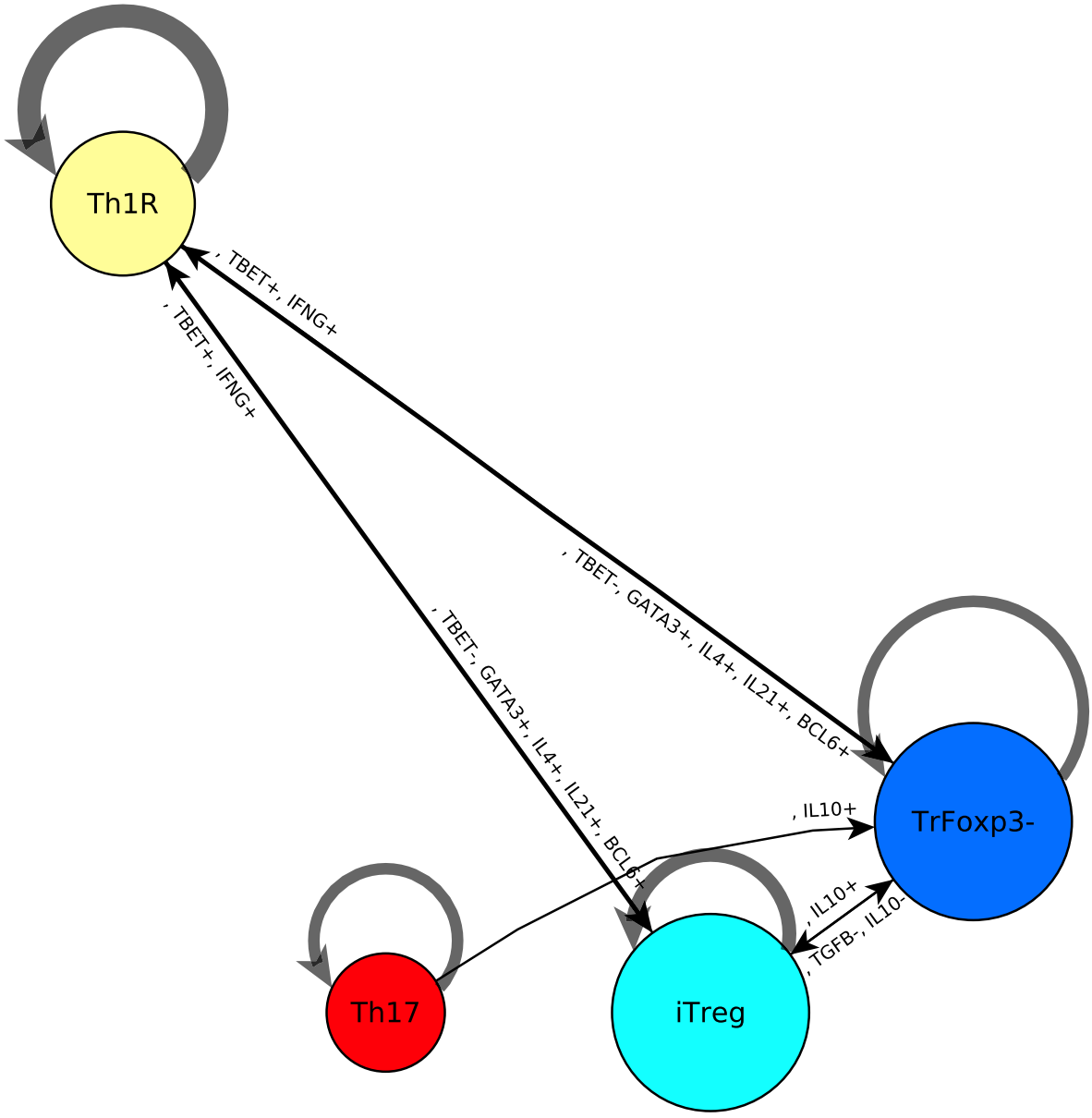
Th2 (IL-4e+ and IL-2e+)



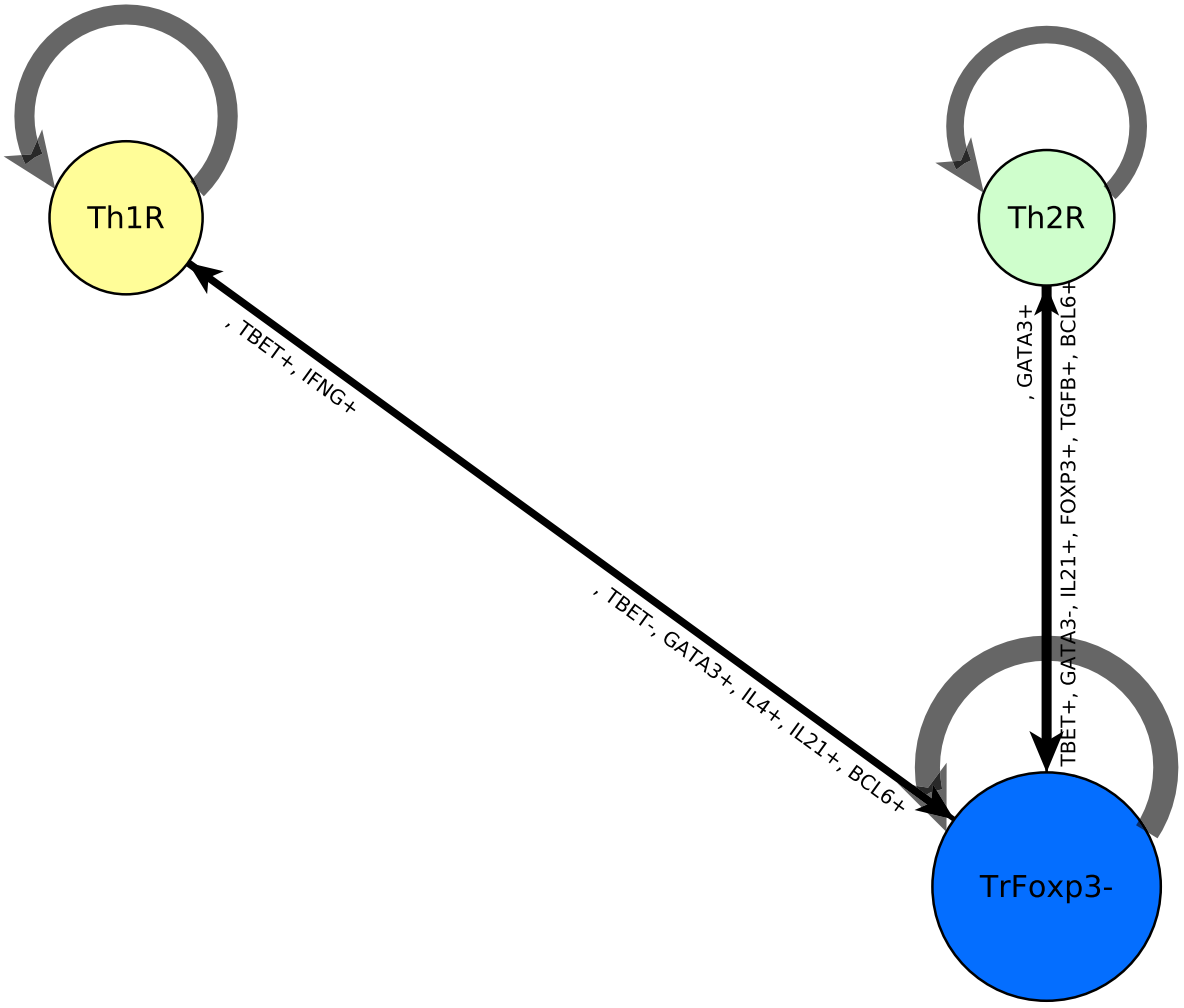
Th17 (IL-21e+ and TGFβe+)



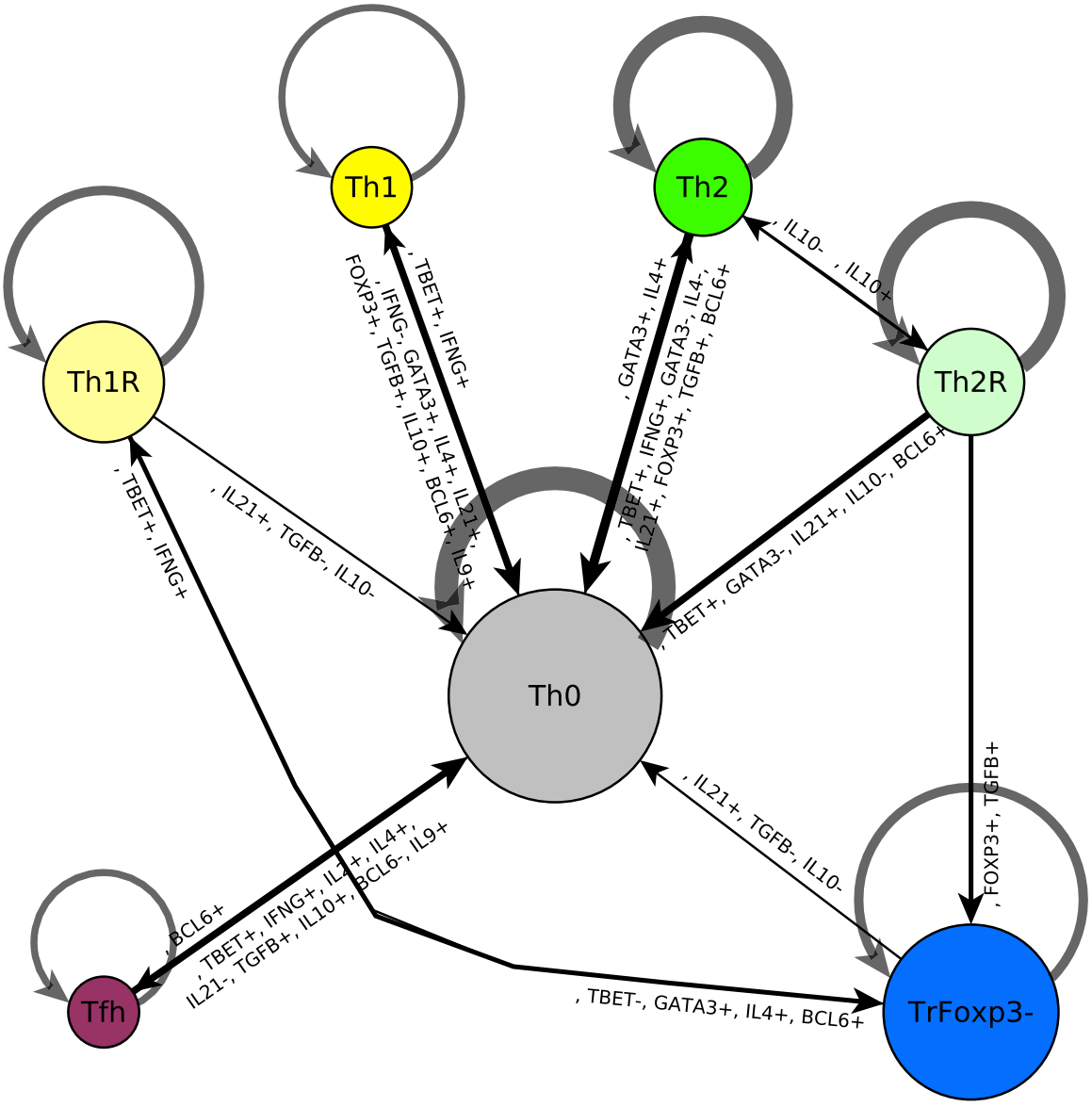
iTreg (TGFβe+ and IL-2e+)



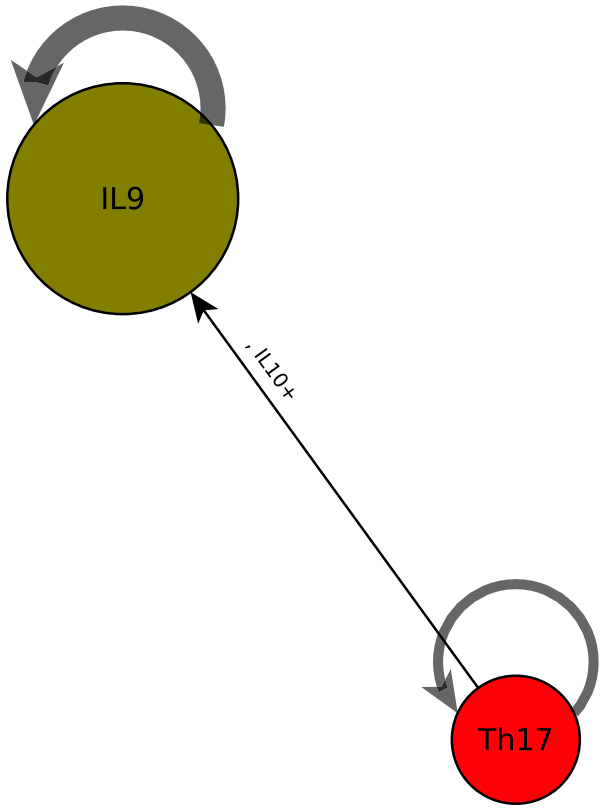
IL-10 (IL-10_e+)


Th1R
Th2R
TrFoxp3-
, TBET+, IFNG+
, TBET-, GATA3+, IL4+, IL21+, BCL6+
, GATA3+
TBET+, GATA3-, IL21+, FOXP3+, TGFB+, BCL6+

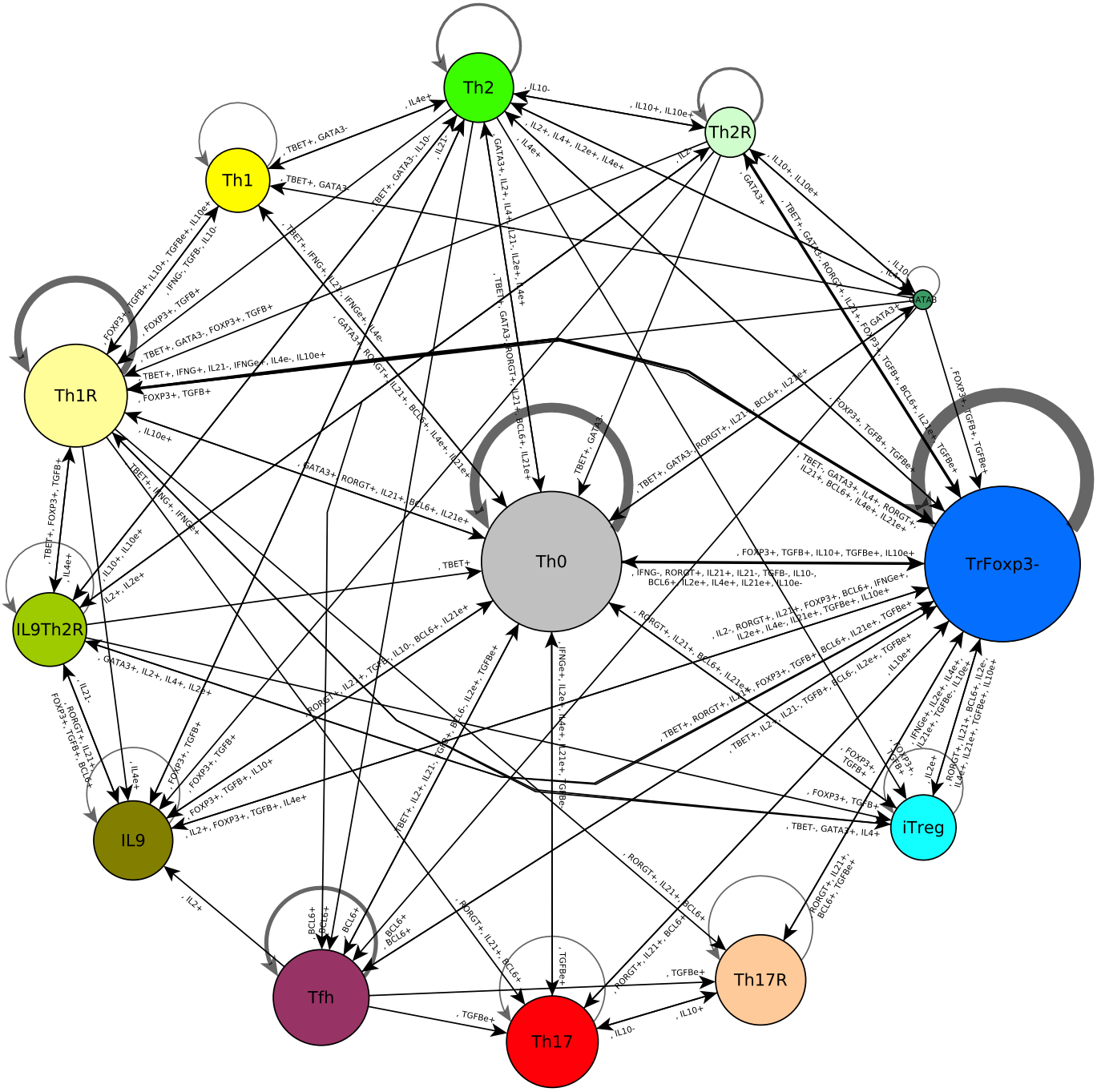
Tfh (IL-21_e+)


Th1
Th2
Th1R
Th2R
Th0
Tfh
TrFoxp3-
, TBET+, IFNG+
, IFNG-, GATA3+, IL4+, IL21+, FOXP3+, TGFB+, IL10+, BCL6+, IL9+
, GATA3+, IL4+
, TBET+, IFNG+, GATA3-, IL4-, IL21+, FOXP3+, TGFB+, BCL6+
, IL10- , IL10+
, TBET+, IFNG+
, IL21+, TGFB-, IL10-
, TBET+, GATA3-, IL21+, IL10-, BCL6+
, IL21+, TGFB-, IL10-
, FOXP3+, TGFB+
, BCL6+
, TBET+, IFNG+, IL21+, IL4+, IL21-, TGFB+, IL10+, BCL6-, IL9+
, TBET-, GATA3+, IL4+, BCL6+

IL-9 (IL-4_e+ and TGFβe)



SOCS-


Th2
Th2R
Th1
Th1R
GATA3
IL9Th2R
Th0
TrFoxp3-
IL9
iTreg
Tfh
Th17
Th17R

Apéndice B

Supplementary: The CD4+ T cell regulatory network mediates inflammatory responses during acute hyperinsulinemia: a simulation study

Supplementary Materials

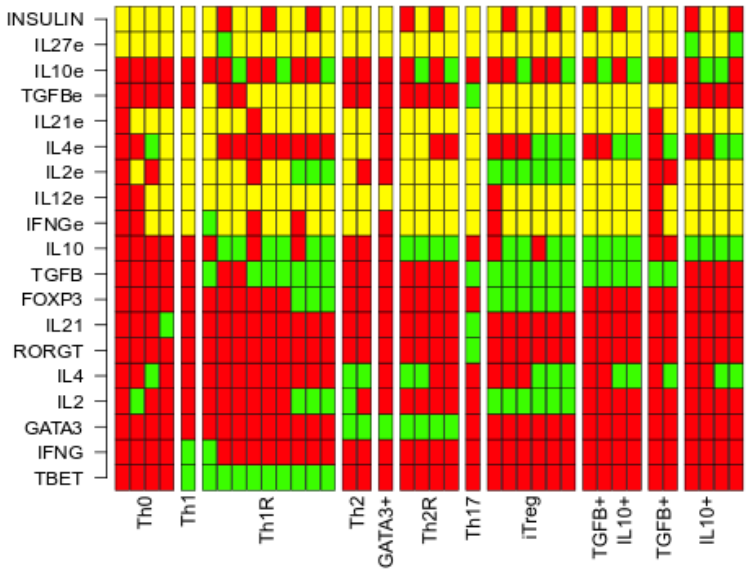


Fig S1: Attractors of the CD4+ T cell regulatory network. Each column corresponds to an attractor. Each node can be active (green) or inactive (red), extrinsic cytokines may be active or inactive (yellow). The following attractors were found in the network: Th0, Th1, Th1R, Th2, GATA3+, Th17, iTreg, TGFβ+IL10+, TGFβ+ and IL10+ regulatory cells. Attractors where labeled according to the active transcription factors and intrinsic cytokines.

Node	Function
TBET	((IFNG | (IL12e & ! (IL21 | IL4 | IL10))) | TBET) & ! (IL4 | GATA3 | IL21)
IFNG	(IFNGe | ((IFNG | TBET) & ! (GATA3 | TGFB))) & ! (IL21 | IL4 | IL10)
GATA3	((IL2 & IL4) | GATA3) & ! (TBET | TGFB | IL21 | IFNG)
IL2	(IL2e | (IL2 & ! FOXP3)) & ! (IFNG | IL21 | (IL10 & ! FOXP3))
IL4	(IL4e | (GATA3 & (IL2 | IL4) & ! TBET)) & ! (IFNG | IL21)
RORGT	(IL21 & TGFB) & ! (TBET | FOXP3 | GATA3)
IL21	(IL21e | IL21 | RORGT) & ! (IFNG | IL4 | IL10 | IL2)
FOXP3	(IL2 & (TGFB | FOXP3)) & ! (IL21 | RORGT)
TGFB	TGFBe | ((TGFB | FOXP3) & ! IL21)
IL10	IL10e | (IL10 & (IFNG | IL21 | TGFB | GATA3 | IL27e) & ! INSULIN)
IFNGe	IFNGe
IL12e	IL12e
IL2e	IL2e
IL4e	IL4e
TGFBe	TGFBe
IL10e	IL10e
IL27e	IL27e

Table S1. Rules of the CD4+ T cell regulatory network.

Labels	Rules
Th0	! (TBET | GATA3 | RORGT | FOXP3 | IL10 | TGFB)
Th1	(TBET & IFNG) & ! (IL10 | TGFB | FOXP3)
TBET+	TBET & ! (IFNG | IL10 | TGFB | FOXP3)
Th1R	TBET & (IL10 | TGFB | FOXP3)
TH2	(GATA3 & IL4) & ! (IL10 | TGFB | FOXP3)
GATA3+	GATA3 & ! (IL4 | IL10 | TGFB | FOXP3)
Th2R	GATA3 & (IL10 | TGFB | FOXP3)
Th17	RORGT & IL21 & ! IL10
RORGT+	RORGT & ! (IL21 | IL10)
iTreg	FOXP3 & TGFB & ! (TBET | GATA3 | RORGT)
IL10+	IL10 & ! (TBET | GATA3 | FOXP3 | RORGT)
TGFB+	TGFB & ! (TBET | GATA3 | FOXP3 | RORGT)

Table S2. Labeling rules of the CD4+ T cell regulatory network.

Apéndice C

Supplementary: The combination of the functionalities of feedback circuits is determinant for the number and size of attractors of molecular networks

Supplementary File: The combination of the functionalities of feedback circuits is determinant for the attractors' number and size in pathway-like Boolean networks

Eugenio Azpeitia[1,*], Stalin Muñoz[2,+], Daniel González-Tokman[3,+], Mariana Esther Martínez-Sánchez[4,5,+], Nathan Weinstein[6,+], Aurélien Naldi[7], Elena R. Álvarez-Buylla[5,8], David A. Rosenblueth[2], and Luis Mendoza[9]

[1]INRIA project-team Virtual Plants, joint with CIRAD and INRA, Montpellier Cedex 5, France.
[2]Instituto de Investigaciones en Matemáticas Aplicadas y en Sistemas, Universidad Nacional Autónoma de México, Apdo. 20-126, 01000 México, D.F., México.
[3]CONACYT, Instituto de Ecología, A. C., Antiguo camino a Coatepec 351, El Haya, 91070. Xalapa, Veracruz, México.
[4]Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, México.
[5]Instituto de Ecología, Universidad Nacional Autónoma de México, México.
[6]ABACUs-Centro de Matemáticas Aplicadas y Cómputo de Alto Rendimiento, Departamento de Matemáticas, Centro de Investigación y de Estudios Avanzados CINVESTAV-IPN, Carretera México-Toluca Km 38.5, La Marquesa, Ocoyoacac, Estado de México, 52740 México.
[7]DIMNP UMR CNRS 5235, Université de Montpellier. France.
[8]Centro de Ciencias de la Complejidad, Universidad Nacional Autónoma de México, México.
[9]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México.
[+]These authors contributed equally to this work.
[*]Corresponding author: eugenio.azpeitia-espinosa@inria.fr

Supplementary Methods

Boolean networks basic definitions

Let $\mathbb{B} = \{0,1\}$ and $\mathbb{N}_{\leq n} = \{1,2,\ldots,n\}$ an initial segment of natural numbers. We define a *synchronous Boolean network with n components* as a function $f\colon \mathbb{B}^n \to \mathbb{B}^n$. The *i-th component of f* is a function $f_i\colon \mathbb{B}^n \to \mathbb{B}$ such that $f_i(x) = f(x)_i$.

A *state* of the network is a tuple $x = (x_1, x_2, \ldots, x_n)$ such that $x \in \mathbb{B}^n$. The dependency of the state on the discrete time parameter t is denoted as $x(t)$ and obeys the evolution rule given by f. That is for all $t \in \mathbb{Z}$

$$x(t+1) = f(x(t))\,. \tag{1}$$

In order to relate a synchronous Boolean network with a molecular network, we interpret each component of a state x as representing the activation state of a particular variable denoting a molecule included in the network. Given a set of variables $V = \{v_1, \ldots, v_n\}$, we define a bijective function $\chi : \mathbb{N}_n^+ \to V$ to relate the components of the state x with their respective variables. The molecule represented by the variable $\chi(i)$ is said to be *active* if $x_i = 1$ and *inactive* otherwise.

For synchronous Boolean networks, an *attractor* is a set of states $A \subseteq \mathbb{B}^n$ where for all $x(0) \in A$ there exist $l > 0$ such that $l = \min_k x(k) = x(0)$ and for all $s \in \mathbb{N}_{\leq l}$, $x(s) \in A$; l is the *size* of the attractor.

For simplicity, we refer to the variable v by its position i in the vector x. For a state $x \in \mathbb{B}^n$ and a variable i we denote as $x \sim i$ the vector resulting from replacing the value of x_i with the complement of x_i.

Given two variables i and j and the update function of variable j, namely f_j, *i activates j* if there exists a pair of network states x, y that differ only in the state of activation of variable i, that is, $y = x \sim i$, $x_i = 0$ and $y_i = 1$, such that $f_j(y) - f_j(x) > 0$. Conversely, *i inhibits j* if there exists a pair of network states x, y that differ only in the state of activation of variable i, that is, $y = x \sim i$, $x_i = 0$ and $y_i = 1$, such that $f_j(y) - f_j(x) < 0$. An *interaction*, denoted as the pair (i, j), $i, j \in \mathbb{N}_{\leq n}$ is *functional* if variable i activates or inhibits node j. Note that according to this definition, it is possible for variable i to both activate and inhibit variable j. A non-functional interaction does not provide useful information about the biological system and it is an accepted convention that interactions, where a molecule activates and inhibits the same gene are scarce in molecular

regulations[1,2]. Thus, we excluded both non-functional regulations and regulations where variable i both activate and inhibit variable j.

Circuits functionality analyses

A feedback circuit is defined as a set of directed interactions forming a closed path. Feedback circuits can be positive or negative. The sign of a circuit is given by the signs of its interactions. A circuit is positive if it has an even number of negative interactions, it is negative otherwise. It is important to note that the sole presence of a circuit in a network does not guarantee the appearance of the corresponding dynamical behavior (i.e., oscillations or multistability). Thus, a circuit is considered functional if at least one combination of the states of external regulators of its members allows all interactions of the circuit to be functional together[3].

In more formal terms, the *functionality context of the interaction* (i,j) of a Boolean network f is the set $\Phi(f,i,j)$ defined by:

$$\Phi(f,i,j) = \{x \mid f_j(x) \neq f_j(x \sim i) \text{ and } x \in \mathbb{B}^n\} \tag{2}$$

For a Boolean network f we say that $\mathscr{G}_f$ is its *structure* or *interaction graph* $\mathscr{G}_f = \langle V, \mathscr{I}_f^+, \mathscr{I}_f^- \rangle$, where: $\mathscr{I}_f^+$ is its set of *positive interactions* defined by

$$\mathscr{I}_f^+ = \{(\chi(i),\chi(j)) \mid x \in \Phi(f,i,j) \text{ and } x_i = f_j(x)\} \tag{3}$$

and $\mathscr{I}_f^-$ is its set of *negative interactions* defined by

$$\mathscr{I}_f^- = \{(\chi(i),\chi(j)) \mid x \in \Phi(f,i,j) \text{ and } x_i \neq f_j(x)\} \tag{4}$$

For a circuit $C = (c_1, c_2, \ldots, c_k)$ (simple cycle) with no shortcuts of an interaction graph $\mathscr{G}_f$, where $c_i \in \mathbb{N}_{\leq n}$, $c_i \neq c_j$ if $i \neq j$, we define the *functionality context of the circuit* C, denoted $\Phi(f,C)$ as follows:

$$\Phi(f,C) = \bigcap_{i=1}^{k} \Phi(f, c_i, c_{(i \bmod k)+1}) \,. \tag{5}$$

The circuit C is *functional* if $\Phi(f,C)$ is not empty.

The *functionality context for a circuit C with shortcuts* $S = \{(c_i, c_j) \mid |(j \bmod k) - i| \neq 1\}$ is defined by:

$$\Phi(f,C,S) = \Phi(f,C) - \bigcup_{(i,j)\in S} \{x \mid x \in \Phi(f,C) \text{ and } x \sim i \notin \Phi(f,C)\} \,. \tag{6}$$

As with the previous case the circuit C is functional if $\Phi(f,C,S)$ is not empty.

The *restricted functionality context* of $\Phi(f,C,S)$ by the set of nodes $P = \{\rho_1, \rho_2, \ldots, \rho_k\}$ having a Boolean constant function (i.e. $\forall x, \forall \rho \in P\, f_\rho(x) = c$ and $c \in \mathbb{B}$) is defined by

$$\Phi(f,C,S)[P] = \{x \mid \forall \rho \in P\, x_\rho = f_\rho(x) \text{ and } x \in \Phi(f,C,S)\} \tag{7}$$

A *functional circuit description* is given by the triple (C,n,ς) where C is a circuit, $n = |\Phi(f,C,S)[P]|$ the cardinality of its restricted functionality context, an its sign $\varsigma \in \mathbb{S}$, where $\mathbb{S} = \{+,-\}$ is the set of signs.

The *combination of the functionality of (feedback) circuits* of a Boolean network f is defined as the set of functional circuit descriptions for all circuits in its interaction graph $\mathscr{G}_f$.

Networks structural and dynamical distances

We define the *adjacency matrix* of a graph $G = \langle V, E \rangle$ with $V = \{v_1, \ldots, v_n\}$ and $E \in V \times V$ as $\mathscr{A}(G) = (a_{ij})$, $(a_{ij}) \in \mathbb{B}^{n \times n}$ with entries satisfying

$$a_{ij} = \begin{cases} 1 & \text{if } (v_i, v_j) \in E \\ 0 & \text{otherwise)} \end{cases} \tag{8}$$

Accordingly, the *structural distance* $D_{\text{str}}(f,g)$ between two Boolean networks f and g is defined by

$$D_{\text{str}}(f,g) = \left\| \mathscr{A}(\langle V, \mathscr{I}_f^+ \cup \mathscr{I}_f^- \rangle) - \mathscr{A}(\langle V, \mathscr{I}_g^+ \cup \mathscr{I}_g^- \rangle) \right\|_1 \tag{9}$$

where $\|\cdot\|_p$ is the matrix entrywise norm defined by

$$\left\|(a_{ij})\right\|_p = \left(\sum_i \sum_j |a_{ij}|^p\right)^{1/p} \tag{10}$$

The *signed structural distance* $D^{\varsigma}_{\mathrm{str}}(f,g)$ between two Boolean networks f and g is defined by

$$D^{\varsigma}_{\mathrm{str}}(f,g) = \sum_{\varsigma_1 \in \mathbb{S}} \sum_{\varsigma_2 \in \mathbb{S}} \#_{\varsigma_1} \cdot \#_{\varsigma_2} \cdot \left\| \mathscr{A}(\langle V, \mathscr{I}_f^{\varsigma_1} \rangle) - \mathscr{A}(\langle V, \mathscr{I}_g^{\varsigma_2} \rangle) \right\|_1 \tag{11}$$

where $\# : \mathbb{S} \to \{-1, 1\}$, is defined by

$$\#_{\varsigma} = \begin{cases} -1 & \text{if } \varsigma = - \\ 1 & \text{if } \varsigma = + \end{cases} \tag{12}$$

The *dynamical distance* between two Boolean networks f and g is defined by

$$D_{\mathrm{dyn}}(f,g) = \sum_{x \in \mathbb{B}^n} \sum_{i \in \mathbb{N}_{\leq n}} |f_i(x) - g_i(x)| \tag{13}$$

We compared each pair of networks A and B of size N using the three distances described above, implementing the necessary algorithms in Python. The dynamical distance clustering analysis was done using the scipy/linkage function (ward)[4] for the dynamical distance. We considered that only distances below a certain threshold where valid edges (0 and 8 for the structural and dynamical distance, respectively). In the resulting networks each node represents a PLN and the edges' weight corresponds to the dynamical or structural distance. We analyzed the network properties using. python/networkx[5].

PLNs simulations, analyses and queries

For section 2.1 and 2.2 PLNs were simulated using R/BoolNet 2.1.1[6]. There were 9×10^3 biologically meaningful 1-PLNs, all of which were analyzed. As for biologically meaningful 2-PLNs, due to their astronomical number, we used samples of sizes between 10^3 and 32.8×10^6 of them to analyze their properties. The combinations of functionalities were analyzed using GINsim[7].

For the sections 2.4 and 2.5, the search of PLNs with the epistasis expected set of attractors (see section 3.3) was done with Griffin[8]. Griffin is a software tool that uses symbolic algorithms for the inference of Boolean networks. Griffin transforms the set of constraints into a Boolean sentence, which in turn using a Tseitin transformation[9,10] is converted into an equisatisfiable conjunctive normal form sentence. This sentence is then provided as an input to a SAT solver, SAT4j[11]. When the solver finds an assignment of the Boolean variables that make the sentence true, this assignment is returned to Griffin. Griffin then decodes the assignment into a set of Boolean functions corresponding to the network dynamics.

Certain biological constraints were added to Griffin to formulate the epistasis queries. First, we use a set of *generalized interactions* which are a set of gene interaction constraints that corresponded to the MUS,OUS and MP interactions. The expected fixed point attractors of the 2-PLNs required partially defined fixed points and the double mutant experiment required multiple genes mutations with partially defined states. All of these biological constraints were transformed by Griffin into the Boolean sentence representing the query. Finally, we prohibited networks that exhibit cyclic trajectories in the state space. As it is computationally intractable to add this constraint a priori, Griffin performs a posteriori refinement of cycles using Dubrova and Teslenko's SAT based algorithm[12]. Any satisfying assignment will be decoded to a biologically meaningful Boolean network.

All the data generated via simulations, analyses and queries are available under request.

Statistical Analyses

All statistical analyses were carried out in R version 3.2.3[13].

To test the relationship between number of attractors and attractor average size we carried out a non-parametric Spearman rank correlation, given that the assumptions of parametric correlation were violated (Fligner-Killeen test for homogeneity of variances $X^2 = 526.784$, $d.f. = 9$, $P < 0.001$).

The differences between the circuits and structural properties in the singles and 2-PLNs were analyzed with generalized linear models (GLM) with Poisson error structure and log link function. In GLMs with overdispersion (overdispersion test $P < 0.05$;[14]) we used models with quasipoisson errors[15]. For analyzing attractor sizes and ratio of positive/negative circuits we used generalized least squares (GLS) to account for heterogeneous variances found by type (Fligner-Killeen test $P < 0.001$)[16,17].

Finally, in order to test the frequency distribution of networks ($N = 6.3X10^7$ networks) of the attractors size and number we used Kolmogorov-Smirnov tests for log-normal, exponential, normal and Poisson distributions implemented in R package nortest. To test for power law distributions we used a bootstrapping procedure with 30 simulations in R package poweRlaw[18].

Statistical analyses results

Attractors properties

The attractors size was significantly larger for 2-PLNs than for 1-PLNs (GLS $F1,18998 = 217.63$,$P < 0.001$; $C.I.95\%$: 2-PLNs$= 1.62 \pm 0.012$; 1-PLNs $= 1.49 \pm 0.012$).

The number of attractors was significantly larger for 2-PLNs than for 1-PLNs (Poisson GLM $z = 103.2$, $d.f. = 1,18998$, $P < 0.001$; $C.I.95\%$: 2PLN $= 6.22 \pm 0.049$; 1-PLNs $= 2.91 \pm 0.035$).

Feedback circuits and PLNs structure

The number of combinations was significantly higher in 2-PLNs than in n–s– 2-PLNs (Poisson GLM: $z = 104.6$, $P < 0.001$, 2-PLNs $1,510 \pm 11.07$, n–s– 2-PLNs 788 ± 7.74).

The number of combinations of functionalities contained in each structure was significantly larger for 2-PLN than for n–s– 2-PLN (Quasipoisson GLM $t = 53.96$, $d.f. = 30768$, $P < 0.001$; $C.I.95\%$: 2PLN $= 14.24 \pm 0.199$; n–s– 2-PLNs $= 2.36 \pm 0.146$).

The mean number of combinations of functionalities contained in each structure was not significantly different for 1-PLNs and 1-n–s– PLNs (Quasipoisson GLM $t = 1.423$, $d.f. = 19$, $P = 0.172$).

The mean number of structures containing the same combination of functionalities was similar between 2-PLNs and n–s– 2-PLNs (Quasipoisson GLM $Res.Dev. = 280865$, $d.f. = 254468$, $P = 0.331$, 2-PLNs 1.46 ± 0.010, n–s– 2-PLNs 1.43 ± 0.044).

Relations

In 1-PLNs, the relationship between the number of attractors and the attractors average size was significant and negative (Poisson GLM: $Res.Dev. = 1139.8$, $d.f. = 8998$, $z = -22.59$, $P < 0.001$). In 1-PLNs, the relationship between the number of attractors and the number of negative feedback circuits was significant and negative (Poisson GLM: $Res.Dev. = 5683.8$, $d.f. = 8998$, $z = -30.45$, $P < 0.001$). The relationship between the number of attractors and the number of positive feedback circuits was significant and positive (Poisson GLM: $Res.Dev. = 5633.5$, $d.f. = 8998$, $z = 34.29$, $P < 0.001$). The relationship between the attractors average size and the number of negative feedback circuits was significant and positive (Poisson GLM: $Res.Dev. = 5428.5$, $d.f. = 8998$, $z = 38.15$, $P < 0.001$). The relationship between the attractors average size and the number of positive feedback circuits was significant and negative (Poisson GLM: $Res.Dev. = 32122$, $d.f. = 8998$, $z = -37.45$, $P < 0.001$).

In 2-PLNs to test the relationship between the number of attractors and the attractors average size we took a random sample of 10,000 networks and carried out a generalized linear model (GLM) with Poisson errors and log link function. We used power tests in G*Power software (version 3.1.9.2) to confirm that this number of sampled networks was enough to achieve high power values in the analyses (higher than 0.90)[19]. In particular, we used two-tailed z-tests for Poisson distributions, using alpha values of 0.05 including the exponential of each model's coefficients as input parameters. When the independent variable was a count (number of attractors) we specified the observed lambda and when the predictor variable was continuous (attractor?s size) we used the observed mean and standard deviation[20,21]. For the relation between positive loops and attractor size we needed to sample 30,000 networks in order to achieve a power of 0.90. We found a significant, negative relation between the number of attractors and the attractors average size (Poisson GLM: $Res.Dev. = 1968.9$, $d.f. = 9998$, $z = -14.00$,$P < 0.001$). The relationship between the number of attractors and the number of negative feedback circuits was significant and negative (Poisson GLM: $Res.Dev. = 146.46$, $d.f. = 9998$, $z = -11.79$, $P < 0.001$). The relationship between the number of attractors and the number of positive feedback circuits was significant and positive (Poisson GLM: $Res.Dev. = 4975.5$, $d.f. = 9998$, $z = 39.60$, $P < 0.001$). The relationship between the attractors average size and the number of negative feedback circuits was significant and positive (Poisson GLM: $Res.Dev. = 9607.10$, $d.f. = 9998$, $z = 39.45$, $P < 0.001$). The relationship between the attractors average size and the number of positive feedback circuits was significant and negative (Poisson GLM: $Res.Dev. = 32122$, $d.f. = 29998$, $z = -19.36$, $P < 0.001$).

For 1-PLN, the connectivity was positively related with the number of attractors ($PoissonGLMz = 3.25$, $P = 0.001$), but not with the size of attractors ($LM : F1,8989 = 0.206$, $P = 0.650$). For 2-PLN, the connectivity was positively related with the number of attractors ($PoissonGLMz = 41.27$, $P < 0.001$), and with the size of attractors ($LM : F1,15936 = 1089.1$, $P < 0.001$).

Distributions

For the number of attractors, the frequency distribution differed significantly from a normal distribution ($D = 0.205$, $P < 0.001$) or from a Poisson distribution ($D = 0.256$, $P < 0.001$). For the attractors size, the frequency distribution differed significantly from a normal distribution ($D = 0.265$, $P < 0.001$), from a Poisson distribution ($D = 0.420$, $P < 0.001$) or from a power law distribution ($KS = 0.013$, $Xmin = 6$, $Scaling = 10.278$, $P < 0.001$). For the number of attractors, the frequency distribution differed significantly from a log-normal distribution ($D = 0.853$, $P < 0.001$) and from an exponential distribution ($D = 0.474$, $P < 0.001$). The attractors size frequency distribution also differed significantly from a log-normal ($D = 0.307$, $P < 0.001$) and an exponential distribution ($D = 0.401$, $P < 0.001$).

References

1. Raeymaekers, L. Dynamics of boolean networks controlled by biologically meaningful functions. *Journal of Theoretical Biology* **218**, 331–341 (2002).
2. Azpeitia, E., Benítez, M., Padilla-Longoria, P., Espinosa-Soto, C. & Alvarez-Buylla, E. R. Dynamic network-based epistasis analysis: boolean examples. *Frontiers in Plant Sciences* **15**, 2:92 (2010).
3. Naldi, A., Thieffry, D. & Chaouiya, C. *Decision Diagrams for the Representation and Analysis of Logical Models of Genetic Networks*, vol. 4695 of *Computational Methods in Systems Biology. Lecture Notes in Computer Science*, 233–247 (Springer Berlin Heidelberg, 2007).
4. Müllner, D. fastcluster: Fast hierarchical, agglomerative clustering routines for r and python. *Journal of Statistical Software* **53**, 1–18 (2013).
5. Hagberg, A., Schult, D. & Swart, P. Networkx reference (2012).
6. Müssel, C., Hopfensitz, M. & Kestler, H. A. Boolnet – an R package for generation, reconstruction and analysis of Boolean networks. *Bioinformatics* **26**, 1378–1380 (2010).
7. Chaouiya, C., Naldi, A. & Thieffry, D. *Logical Modelling of Gene Regulatory Networks with GINsim*, 463–479 (Springer New York, New York, NY, 2012).
8. Rosenblueth, D. A., Muñoz, S., Carrillo, M. & Azpeitia, E. Inference of Boolean Networks from Gene Interaction Graphs Using a SAT Solver. In *Algorithms for Computational Biology*, 235–246 (Springer International Publishing, 2014).
9. Prestwich, S. D. CNF encodings. *Handbook of Satisfiability* **185**, 75–97 (2009).
10. Tseitin, G. S. On the complexity of derivation in propositional calculus. In *Automation of reasoning*, 466–483 (Springer, 1983).
11. Le Berre, D. & Parrain, A. The sat4j library, release 2.2, system description. *Journal on Satisfiability, Boolean Modeling and Computation* **7**, 59–64 (2010).
12. Dubrova, E. & Teslenko, M. A SAT-based algorithm for finding attractors in synchronous Boolean networks. *IEEE/ACM Transactions on Computational Biology and Bioinformatics (TCBB)* **8**, 1393–1399 (2011).
13. R Development Core Team. *R: a Language and Environment for Statistical Computing* (Austria, 2015).
14. Kleiber, C. & Zeileis, A. *Applied Econometrics with R* (New York, U.S.A, 2008).
15. Crawley, M. J. *The R Book* (London, U.K., 2007), 1st edn.
16. Venables, W. N. & Ripley, B. D. *Modern Applied Statistics with S* (New York, U.S.A, 2002), fourth edn.
17. Zuur, A. F., Ieno, E. N., Walker, A. A., N. J.and Saveliev & Smith, G. M. *Mixed Effects Models and Extensions in Ecology with R* (New York, U.S.A, 2009).
18. Gillespie, C. S. Fitting heavy tailed distributions: The poweRlaw package. *Journal of Statistical Software* **64**, 1–16 (2015). URL http://www.jstatsoft.org/v64/i02/.
19. Ellis, P. D. *The Essential Guide to Effect Sizes* (Cambridge University Press, 2010).
20. Faul, F., Erdfelder, E., Lang, A.-G. & Buchner, A. G*power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* **39**, 175–191 (2007).
21. Faul, F., Erdfelder, E., Buchner, A. & Lang, A.-G. Statistical power analyses using g*power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods* **41**, 1149–1160 (2009).

Table S1. 2-PLNs contrasts the ratio and total number of feedback circuits between the different groups Results from linear models (circuits ratio) and generalized linear model with Poisson distribution (total circuits). In the upper part of the diagonal for each percentage used are p-values for circuits ratio and in the lower part of the diagonal are p-values for total circuits, corresponding to Table 1.

2-PLNs					
Percentage	PLN group	n–s–	n–s+	n+s–	n+s+
70%	n–s–	—	< 0.001	< 0.001	< 0.001
	n–s+	< 0.001	—	< 0.001	< 0.001
	n+s–	< 0.001	< 0.001	—	< 0.001
	n+s+	< 0.001	< 0.001	< 0.001	—
80%	n–s–	—	< 0.001	< 0.001	< 0.001
	n–s+	< 0.001	—	< 0.001	< 0.001
	n+s–	< 0.001	< 0.001	—	< 0.001
	n+s+	< 0.001	< 0.001	< 0.001	—
90%	n–s–	—	< 0.001	< 0.001	NA
	n–s+	< 0.001	—	< 0.001	NA
	n+s–	< 0.001	< 0.001	—	NA
	n+s+	NA	NA	NA	—
95%	n–s–	—	< 0.001	< 0.001	NA
	n–s+	< 0.001	—	< 0.001	NA
	n+s–	< 0.001	< 0.001	—	NA
	n+s+	NA	NA	NA	—
99%	n–s–	—	< 0.001	< 0.001	NA
	n–s+	< 0.001	—	< 0.001	NA
	n+s–	< 0.001	< 0.001	—	NA
	n+s+	NA	NA	NA	—

Table S2. 1-PLNs contrasts of the ratio and total number of feedback circuits between the different groups In the upper part of the diagonal are p-values of contrasts from linear models for circuits ratio (even when variances were heterogeneous in some cases, modifying variance structure with GLS did not improve model fit); in the lower part of the diagonal are p-values of contrasts from Poisson GLMs for total circuits, corresponding to Table 1. 70, 95 and 99% results are not presented due to the lack of data. NA=data not available to carry out contrasts.

1-PLNs					
Percentage	PLN group	n–s–	n–s+	n+s–	n+s+
80%	n–s–	—	< 0.001	< 0.001	NA
	n–s+	0.812	—	< 0.001	NA
	n+s–	0.044	0.003	—	NA
	n+s+	NA	NA	NA	—
90%	n–s–	—	< 0.001	< 0.001	NA
	n–s+	0.372	—	< 0.001	NA
	n+s–	0.002	< 0.001	—	NA
	n+s+	NA	NA	NA	—

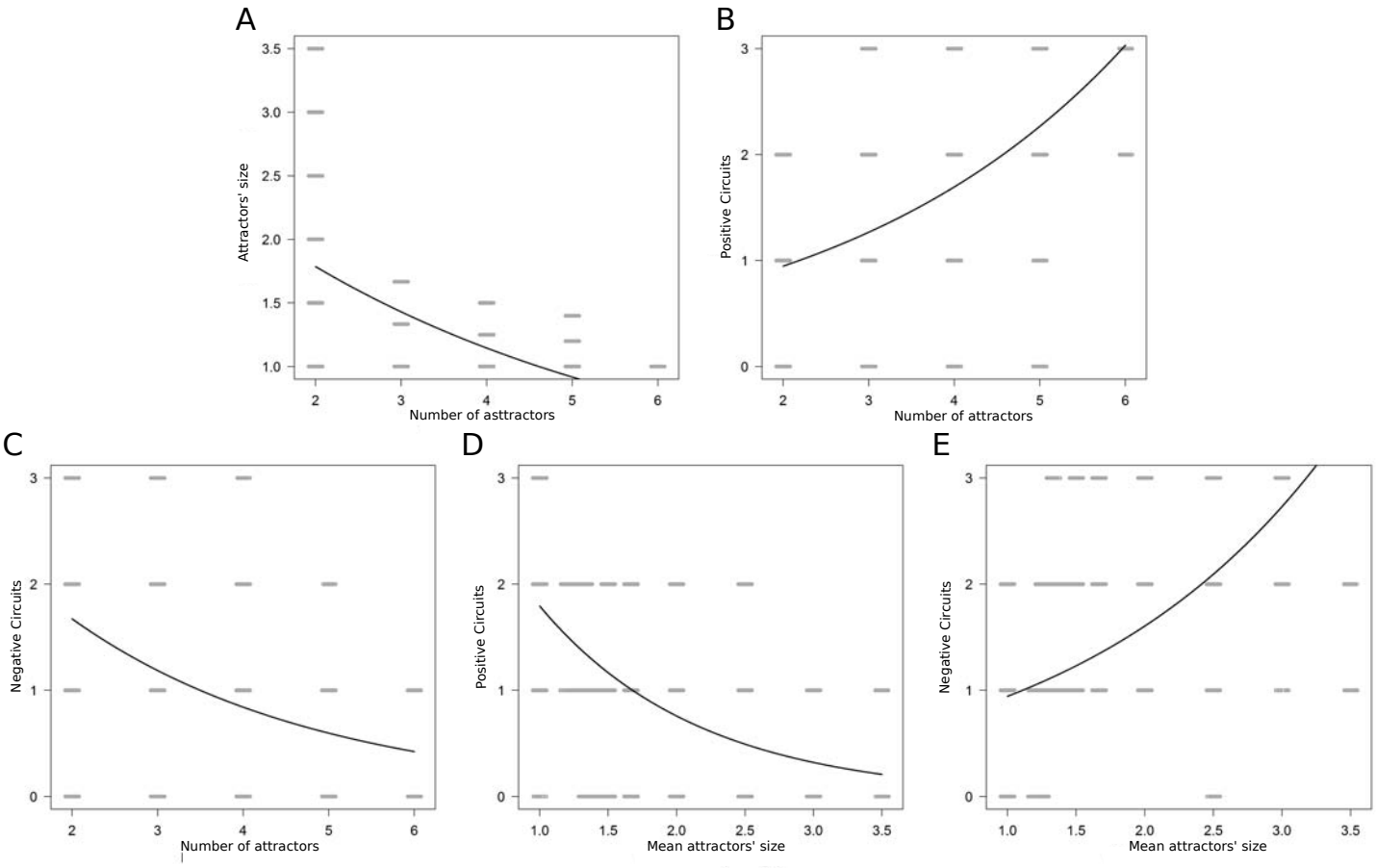
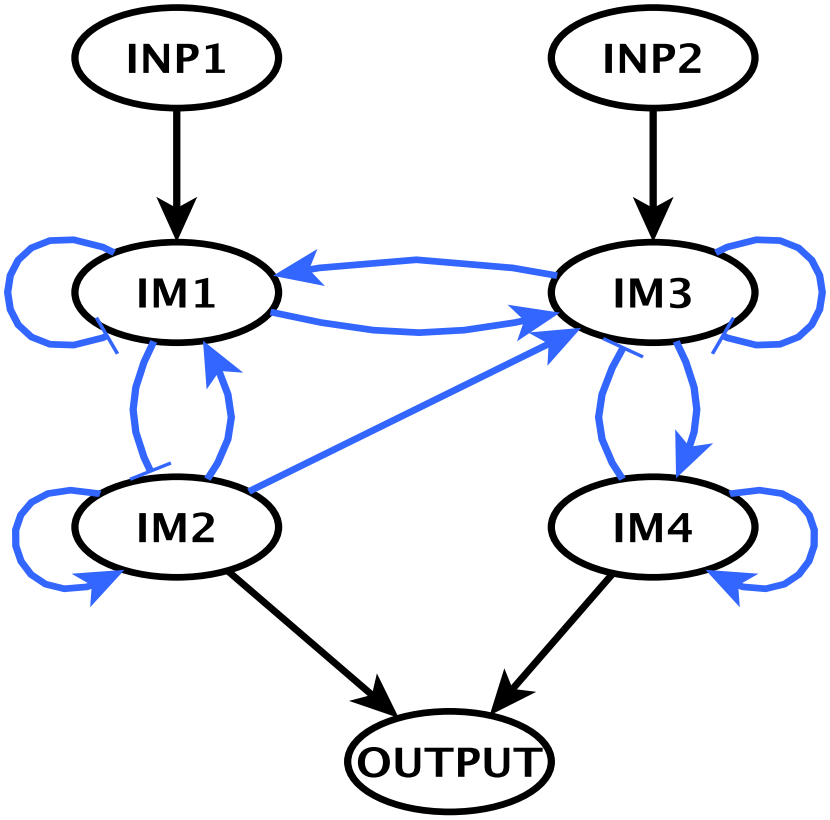


Figure S1. 1-PLNs properties.(A) Number of attractor vs. attractors mean size. (B) and (C) number of attractors vs. quantity of negative and positive feedback circuits, respectively. (D) and (E) size of attractors vs. quantity of negative and positive feedback circuits, respectively. As observed, negative and positive feedback circuits have opposite effects, just as in 2-PLNs. Each point represents a single 2-PLN data. Points are displaced in the X axis only for visual purpose. The lines are predicted by Poisson GLMs.



IM1 = (INP1 | IM2 | ! IM1) & IM3
IM2 = ! IM1 & IM2
IM3 = INP2 & IM1 & IM2 & ! IM3 & ! IM4
IM4 = IM3 | IM4
OUTPUT = IM4 & IM2

Figure S2. Example of a n+s– 2-PLN with an low positive/negative feedback circuits ratio. Interaction graph of a 2-PLN that has three positive and five negative feedback circuits formed with the blue interactions. Because, this PLN has more negative (all functional) than positive feedback circuit (two of them functional), its positive/negative ratio (0.6 considering all feedback circuits and 0.4 considering only the functional ones) is lower than the mean feedback circuits ratio on n–s+ 2-PLNs using any percentage. Thus, we would not expect a n+s– behavior. Anyhow, this network produces 16 fixed-point attractors (i.e., it is a n+s– 2-PLN) using the Boolean functions below (the inputs follow the identity function). &, | and ! stand for the AND, OR and NOT logical operators.

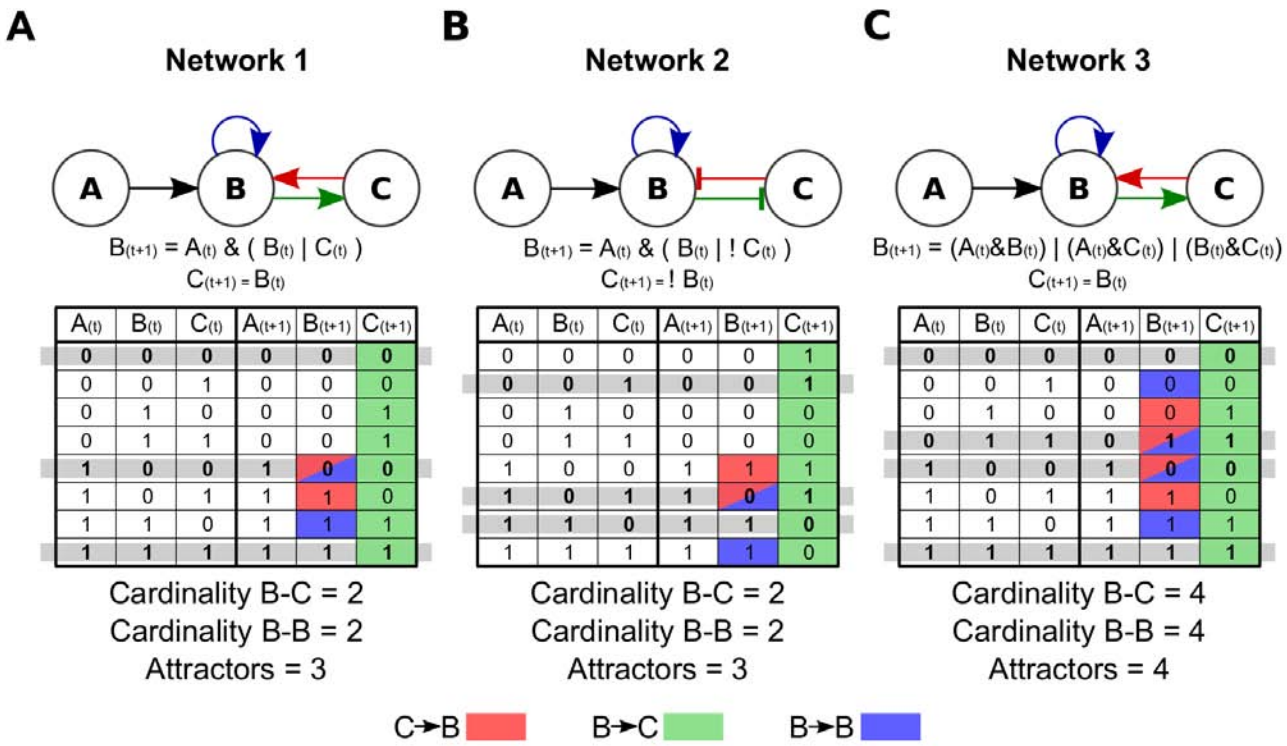
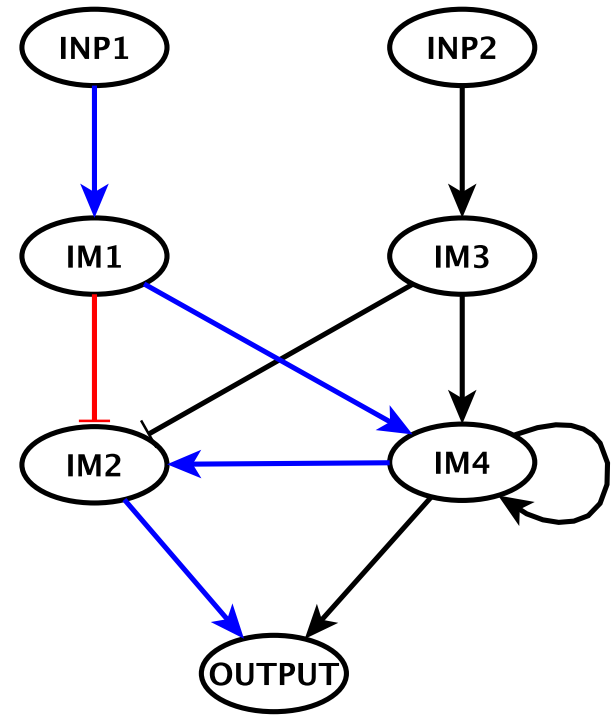
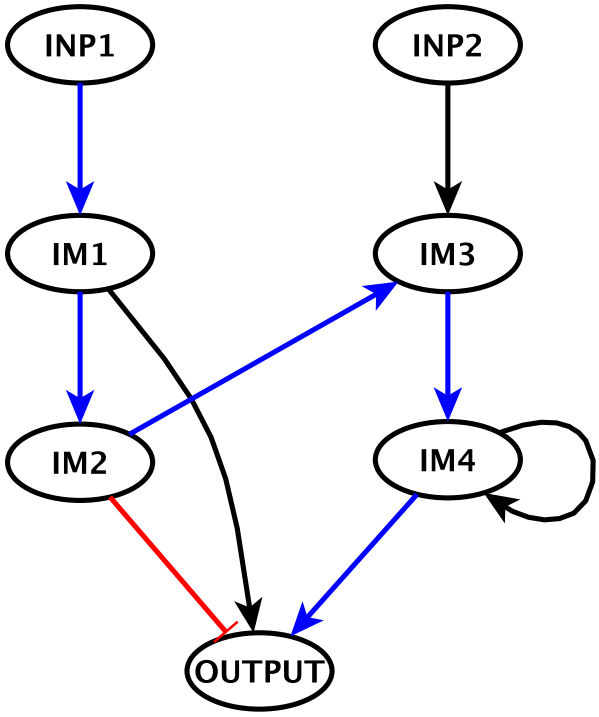


Figure S3. Some properties of the combinations of functionalities. (A-C) Above, the interaction graphs of three networks with two positive feedback circuits. The red, green and blue arrows highlight the interactions forming the circuits. In the middle, the Boolean functions used to solve these networks (*A* follows the identity function). Below, the state diagrams of the networks. The red, green and blue color, highlight the lines where the interactions are functional in the state diagram. The attractors are highlighted with grey. (A) and (B) Network 1 and 2 have the same combination of functionalites comprising two positive feedback circuits, each with a cardinality of 2. (C) Network 3 has a different combination of functionalites of two positive feedback circuits, each with a cardinality of 4. Notice that the same network structure (Network 1 and network 3) can produce different combinations of functionalities, and that different network structures (network 1 and network 2) can produce the same combination of functionalities. Observe that because both circuits share B variable, a change in the cardinality of one circuit can modify the cardinality of the other circuit. Finally, remark that the networks with the same combination of functionality produce the same number of attractors, while networks with different combinations of functionalities produce a different number of attractors. Similar cases exist for the size of the attractors. &, | and ! stand for the AND, OR and NOT logical operators.



IM1 = INP1
IM2 = ! IM1 & ! IM3 | IM4
IM3 = INP2
IM4 = IM3 & IM4 | IM1 & IM4 | IM1 & IM3
OUTPUT = IM2 & IM4



IM1 = INP1
IM2 = IM1
IM3 = INP2 & IM2
IM4 = IM3 | IM4
OUTPUT = IM4 & (IM2 | ! IM2)

Figure S4. Two examples of wrongly inferred interactions using epistasis analysis. Examples of the cases where the interaction from IM1 to IM2 (A) and the interaction from IM2 to OUTPUT (B) are wrongly inferred. In both cases the expected pathway variant is ++. The orange edge is the incorrect inferred interaction and the blue edges are alternative pathways that contains the expected signs of interactions between INP1, IM1, IM2 and OUTPUT with some extra intermediary interactions. Below the Boolean functions for each of these PLNs (the inputs value is fixed to 1). &, | and ! stand for the AND, OR and NOT logical operators.

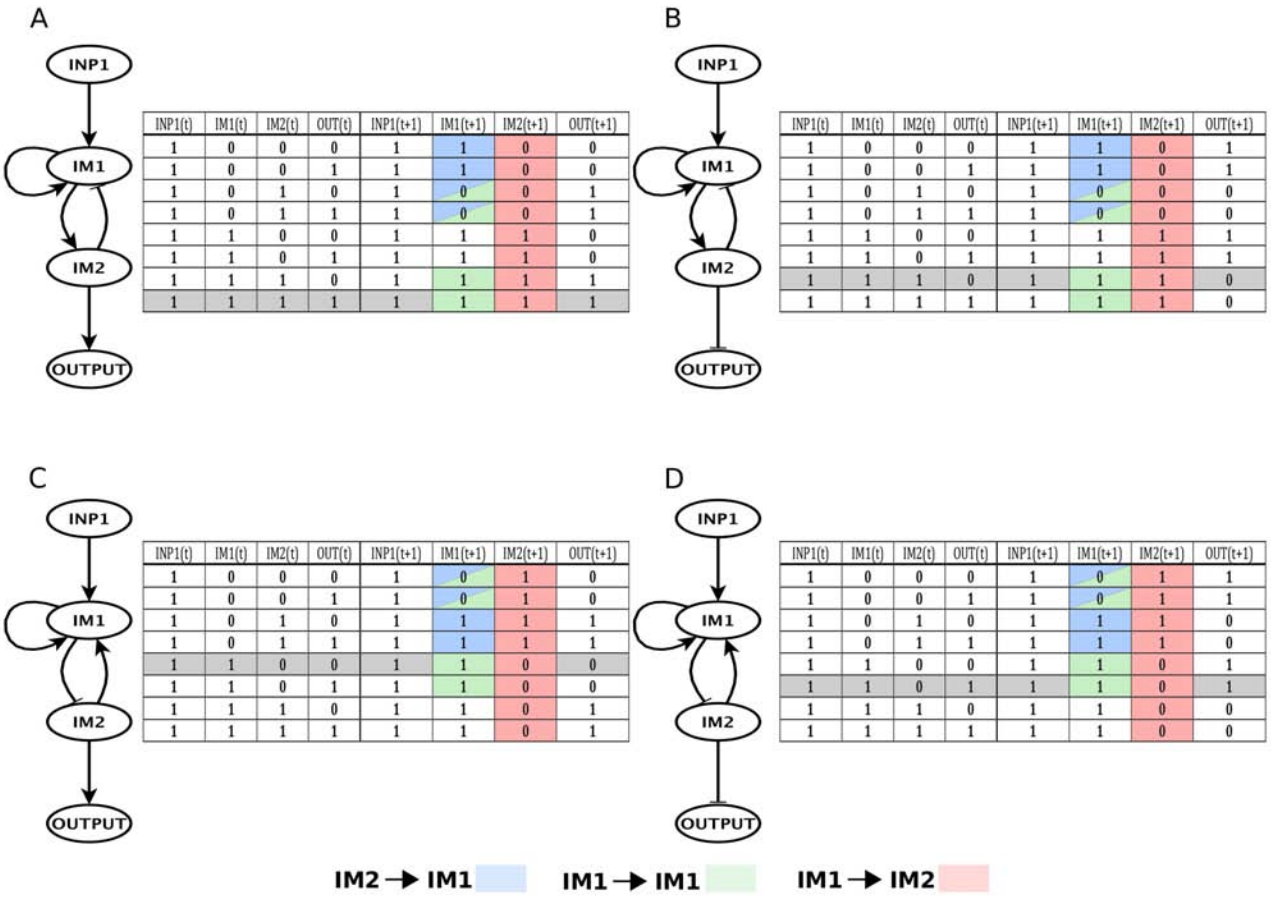


Figure S5. Examples of 1-PLNs with the same combination but different pathway variant. 1-PLNs interaction graphs containing and producing the expected attractors of the (A) ++, (B) +–, (C) –+ and (D) – – pathway variants. Interestingly, all these 1-PLNs have the same combination of functionalities, comprising a positive feedback circuit from IM1 to IM1 with a cardinality of four, and a negative feedback circuit between IM1 and IM2 with a cardinality of four. In the state diagrames, green, blue and pink colors, highlight the lines where the interactions are functional. The attractors are highlighted with grey.

Bibliografía

[1] John J O'Shea. Helper T-Cell Differentiation and Plasticity. In William E Paul, editor, *Fundamental Immunology*. Lippincott Williams & Wilkins, 7th edition, 2013.

[2] Jinfang Zhu, Hidehiro Yamane, and William E Paul. Differentiation of effector CD4 T cell populations (*). *Annual review of immunology*, 28:445–89, jan 2010.

[3] John J. O'Shea, Massimo Gadina, Yuka Kanno, John J O Shea, Massimo Gadina, and Yuka Kanno. Cytokine signaling: birth of a pathway. *The Journal of Immunology*, 187(11):5475–8, nov 2011.

[4] Akihiko Yoshimura, Mayu Suzuki, Ryota Sakaguchi, Toshikatsu Hanada, and Hideo Yasukawa. SOCS, Inflammation, and Autoimmunity. *Frontiers in Immunology*, 3(March):1–9, 2012.

[5] Akihiko Yoshimura, Tetsuji Naka, and Masato Kubo. SOCS proteins, cytokine signalling and immune regulation. *Nature reviews. Immunology*, 7(6):454–65, jun 2007.

[6] Sandra Galic, Nirupa Sachithanandan, Thomas W Kay, and Gregory R Steinberg. Suppressor of cytokine signalling (SOCS) proteins as guardians of inflammatory responses critical for regulating insulin sensitivity. *Biochem. J*, 461:177–188, 2014.

[7] Camille A. Knosp and James A. Johnston. Regulation of CD4+ T-cell polarization by suppressor of cytokine signalling proteins. *Immunology*, 135(2):101–11, feb 2012.

[8] Wilfried Ellmeier and Ichiro Taniuchi. *Transcriptional Control of Lineage Differentiation in Immune Cells*, volume 381. 2014.

[9] Yuka Kanno, Golnaz Vahedi, Kiyoshi Hirahara, Kentner Singleton, John J O'Shea, John J O Shea, John J O'Shea, John J O Shea, and John J O'Shea. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annual review of immunology*, 30:707–731, jan 2012.

[10] M J Warrell, Andreas Leimbach, Jörg Hacker, and Ulrich Dobrindt. Intradermal Rabies Vaccination: The Evolution and Future of Pre- and Post-exposure Prophylaxis. *Current topics in microbiology and immunology*, 358(January):3–32, 2013.

[11] Nancie J MacIver, Ryan D Michalek, and Jeffrey C Rathmell. Metabolic Regulation of T Lymphocytes. *Annual review of immunology*, 31(December 2012):259–283, jan 2013.

[12] Yuka Kanno Michael Bonelli, Han-Yu Shih, Kiyoshi Hirahara, Kentner Singelton, Arian Laurence, Amanda Poholek, Tim Hand, Yohei Mikami, Golnaz Vahedi and John J. O'Shea. Helper T Cell Plasticity: Impact of Extrinsic and Intrinsic Signals on Transcriptomes and Epigenomes. *Current topics in microbiology and immunology*, 358(January):3–32, 2013.

[13] A Ndrew Y Ates A, C Laudia B Ergmann Ab, J L E O V A N H Emmen B, J Aroslav S Tark, R Obin C Allard, a Yates, C Bergmann, J L Van Hemmen, J Stark, and R Callard. Cytokine-modulated regulation of helper T cell populations. *Journal of theoretical biology*, 206(4):539–60, oct 2000.

[14] Adrian Liston and Daniel H D Gray. Homeostatic control of regulatory T cell diversity. *Nature reviews. Immunology*, 14(3):154–65, 2014.

[15] H. J. Van den Ham, A. C. Andeweg, and R. J. de Boer. Induction of appropriate Th-cell phenotypes: Cellular decision-making in heterogeneous environments. *Parasite Immunology*, 35(11):318–330, 2013.

[16] John J.J. John J O'Shea, William E. W.E. Paul, and John J O Shea. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science (New York, N.Y.)*, 327(5969):1098–102, feb 2010.

[17] Brigitta Stockinger Kenneth M Murphy, KM Kenneth M Kenneth M Murphy, Brigitta Stockinger, and Author Manuscript. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nature immunology*, 11(8):674–680, aug 2010.

[18] Michel DuPage and Jeffrey A. Bluestone. Harnessing the plasticity of CD4+ T cells to treat immune-mediated disease. *Nature Reviews Immunology*, 16(3):149–163, feb 2016.

[19] JA A J.A. A J A Bluestone, CR R C.R. R CR R C.R. R Mackay, Jj J.J. JJ J J O'Shea, and Brigitta Stockinger. The functional plasticity of T cell subsets. *Nature Reviews ...*, 9(11):811–816, 2009.

[20] Markus Kleinewietfeld and David A Hafler. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Seminars in immunology*, 25(4):305–12, nov 2013.

[21] LiLi LL Lili Xu, Atsushi Kitani, Ivan Fuss, Warren Strober, and Email Alerts. Cutting Edge: Regulatory T Cells Induce CD4+CD25-Foxp3- T Cells or Are Self-Induced to Become Th17 Cells in the Absence of Exogenous TGF-B. *The Journal of Immunology*, 178(11):6725–6729, jun 2012.

[22] Yun Kyung Lee, Ryuta Mukasa, Robin D. Hatton, and Casey T. Weaver. Developmental plasticity of Th17 and Treg cells. *Current opinion in immunology*, 21(3):274–80, jun 2009.

[23] Margarida Saraiva and Anne O'Garra. The regulation of IL-10 production by immune cells. *Nature reviews. Immunology*, 10(3):170–81, mar 2010.

[24] Ashleigh Howes, Philippa Stimpson, Paul Redford, Leona Gabrysova, and Anne O'Garra. Interleukin-10: Cytokines in Anti-inflammation and Tolerance. In *Cytokine Frontiers: Regulation of Immune Responses in Health and Disease*, volume 6, pages 327–352. 2014.

[25] Ekaterina A. Ivanova and Alexander N. Orekhov. T Helper lymphocyte subsets and plasticity in autoimmunity and cancer: An overview, 2015.

[26] Markus Feuerer, Laura Herrero, Daniela Cipolletta, Afia Naaz, Jamie Wong, Jongsoon Lee, Allison Goldfine, Christophe Benoist, Steven Shoelson, and Diane Mathis. Fat Treg cells: a liaison between the immune and metabolic systems. *Nature medicine*, 15(8):930–939, 2009.

[27] Markus Feuerer, Laura Herrero, Daniela Cipolletta, Afia Naaz, Jamie Wong, Ali Nayer, Jongsoon Lee, Allison B Goldfine, Christophe Benoist, Steven Shoelson, and Diane Mathis. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nature medicine*, 15(8):930–9, aug 2009.

[28] Frühbeck G. Rodríguez A, Ezquerro S, Méndez-Giménez L, Becerril S. Revisiting the adipocyte: a model for integration of cytokine signaling in the regulation of energy metabolism. *Am J Physiol Endocrinol Metab.*, 309(8):E691–714, 2015.

[29] Jonathan M. Han, Scott J. Patterson, Madeleine Speck, Jan a. Ehses, and Megan K. Levings. Insulin inhibits IL-10-mediated regulatory T cell function: implications for obesity. *Journal of immunology (Baltimore, Md. : 1950)*, 192(2):623–9, jan 2014.

[30] Dávid Deritei, William C Aird, Mária Ercsey-Ravasz, and Erzsébet Ravasz Regan. Principles of dynamical modularity in biological regulatory networks. *Scientific reports*, 6:21957, jan 2016.

[31] S.A. Kauffman. Metabolic stability and epigenesis in randomly constructed genetic nets. *Journal of Theoretical Biology*, 22(3):437–467, mar 1969.

[32] Eugenio Azpeitia, Mariana Benítez, Pablo Padilla-Longoria, Carlos Espinosa-Soto, and Elena R Alvarez-Buylla. Dynamic network-based epistasis analysis: boolean examples. *Frontiers in plant science*, 2:92, jan 2011.

[33] Réka Albert and Juilee Thakar. Boolean modeling: a logic-based dynamic approach for understanding signaling and regulatory networks and for making useful predictions. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 6(5):353–369, sep 2014.

[34] Isaac Crespo, Thanneer M Perumal, Wiktor Jurkowski, and Antonio del Sol. Detecting cellular reprogramming determinants by differential stability analysis of gene regulatory networks. *BMC Systems Biology*, 7(1):140, 2013.

[35] Wassim Abou-Jaoudé, Pedro T Monteiro, Aurélien Naldi, Maximilien Grandclaudon, Vassili Soumelis, Claudine Chaouiya, and Denis Thieffry. Model checking to assess T-helper cell plasticity. *Frontiers in bioengineering and biotechnology*, 2:86, jan 2014.

[36] Aurelien Naldi, Denis Thieffry, and Claudine Chaouiya. Decision diagrams for the representation and analysis of logical models of genetic networks. *Computational Methods in Systems Biology*, 4695(i):233–247, 2007.

[37] Jean-Paul Paul Comet, Mathilde Noual, Adrien Richard, Julio Aracena, Laurence Calzone, Jacques Demongeot, Marcelle Kaufman, Aurélien Naldi, El Houssine Snoussi, and Denis Thieffry. On Circuit Functionality in Boolean Networks. *Bulletin of Mathematical Biology*, 75(6):906–919, jun 2013.

[38] El Houssine Snoussi. Necessary Conditions for Multistationarity and Stable Periodicity, mar 1998.

[39] Julio Aracena. Maximum number of fixed points in regulatory Boolean networks. *Bulletin of Mathematical Biology*, 70(5):1398–1409, jul 2008.

[40] Andreas Wagner. Distributed robustness versus redundancy as causes of mutational robustness. *Bioessays*, 27(2):176–188, 2005.

[41] Karl J Niklas, Sarah E Bondos, A Keith Dunker, and Stuart A Newman. Rethinking gene regulatory networks in light of alternative splicing, intrinsically disordered protein domains, and post-translational modifications. *Frontiers in cell and developmental biology*, 3(February):8, 2015.

[42] Gustavo Arellano, Julián Argil, Eugenio Azpeitia, Mariana Benítez, Miguel Carrillo, Pedro Góngora, David A Rosenblueth, and Elena R Alvarez-Buylla. .ªntelope": a hybrid-logic model checker for branching-time Boolean GRN analysis. *BMC bioinformatics*, 12(1):490, jan 2011.

[43] William E. Paul. Fundamental Immunology, Pub, 2012.

[44] Alexander Y Rudensky. Regulatory T cells and Foxp3. *Immunological reviews*, 241(1):260–8, may 2011.

[45] Ronald N Germain. Special regulatory T-cell review: A rose by any other name: from suppressor T cells to Tregs, approbation to unbridled enthusiasm. *Immunology*, 123(1):20–7, jan 2008.

[46] Gerd R Burmester and Antonio Pezzutto. *Color Atlas of Immunology.* Georg Thieme Verlag, 2003.

[47] Michael A Rieger and Timm Schroeder. Hematopoiesis. *Cold Spring Harbor Laboratory Press*, pages 1–18, 2016.

[48] Laura M Calvi and Daniel C Link. Perspectives The hematopoietic stem cell niche in homeostasis and disease. *Blood*, 126(22):2443–2451, 2015.

[49] Sergei Doulatov, Faiyaz Notta, Elisa Laurenti, and John E. Dick. Hematopoiesis: A human perspective. *Cell Stem Cell*, 10(2):120–136, 2012.

[50] Juan-Manuel Anaya, Yehuda Shoenfeld, Paula A Correa, Mario Garcia-Carrasco, and Ricard Cervera. *Autoinmunidad y Enfermedad Autoinmune*. Corporacion para investigaciones biologicas, 2005.

[51] Chan-Wang J Lio and Chyi-Song Hsieh. Becoming self-aware: the thymic education of regulatory T cells. *Current opinion in immunology*, 23(2):213–9, may 2011.

[52] Steven Z Josefowicz, Li-Fan F Lu, and Alexander Y Rudensky. Regulatory T cells: mechanisms of differentiation and function. *Annual review of immunology*, 30:531–64, jan 2012.

[53] C Janeway and K Murphy. Janeway's Immunobiology (Gardland Science). 2011.

[54] A K Abbas and A H Lichtman. *Inmunologia celular y molecular*. Elsevier, 2004.

[55] V R Mosmann, H Cherwinski, M W Bond, M A Giedlin, and R L Coffman. Two Types Of Murine Helper T Cell Clone. *The Journal of Immunology*, 136(7):2348–2357, 1986.

[56] Atkins C J Locksley R M Stockinger B Veldhoen M Hocking RJ, Marc Veldhoen, Richard J Hocking, Christopher J Atkins, Richard M Locksley, and Brigitta Stockinger. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*, 24(2):179–189, feb 2006.

[57] Ritobrata Goswami and Mark H Kaplan. A brief history of IL-9. *Journal of immunology (Baltimore, Md. : 1950)*, 186(6):3283–8, mar 2011.

[58] Yong Lu, Sungyoul Hong, Haiyan Li, Jungsun Park, Bangxing Hong, Lijuan Wang, Yuhuan Zheng, Zhiqiang Liu, Jingda Xu, Jin He, Jing Yang, Jianfei Qian, and Qing Yi. Th9 cells promote antitumor immune responses in vivo. *The Journal of clinical investigation*, 122(11):4160–71, nov 2012.

[59] Edgar Schmitt, Matthias Klein, and Tobias Bopp. Th9 cells, new players in adaptive immunity. *Trends in immunology*, 35(2):61–8, feb 2014.

[60] Mark H Kaplan. Th9 cells: differentiation and disease. *Immunological reviews*, 252(1):104–15, mar 2013.

[61] Stefanie Eyerich, Kilian Eyerich, Davide Pennino, Teresa Carbone, Francesca Nasorri, Sabatino Pallotta, Francesca Cianfarani, Teresa Odorisio, Claudia Traidl-Hoffmann, Heidrun Behrendt, Stephen R. Durham, Carsten B. Schmidt-Weber, and Andrea Cavani. Th22 cells

represent a distinct human T cell subset involved in epidermal immunity and remodeling. *Journal of Clinical Investigation*, 119(12):3573–85, nov 2009.

[62] Hideki Fujita. The role of IL-22 and Th22 cells in human skin diseases. *Journal of dermatological science*, 72(1):3–8, oct 2013.

[63] Scott Commins, John W Steinke, and Larry Borish. The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *The Journal of allergy and clinical immunology*, 121(5):1108–11, may 2008.

[64] Patrick Schaerli, Katharina Willimann, Alois B. Lang, Martin Lipp, Pius Loetscher, and Bernhard Moser. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *The Journal of Experimental Medicine*, 192(11):1553–62, 2000.

[65] Kremmer E Ellwart J Sallusto F et al Breitfeld D Ohl L, Kremmer E Ellwart J Sallusto F et al Breitfeld D Ohl L, D Breitfeld, L Ohl, E Kremmer, J Ellwart, F Sallusto, M Lipp, and R Förster. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *The Journal of experimental medicine*, 192(11):1545–52, dec 2000.

[66] Shane Crotty. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*, 29(1):621–663, apr 2011.

[67] Carola G. CG Vinuesa, Sidonia Fagarasan, and Chen Dong. New territory for T follicular helper cells. In *Immunity*, volume 39, pages 417–420. Elsevier Ltd, sep 2013.

[68] Jennifer L Cannons, Kristina T Lu, and Pamela L Schwartzberg. T follicular helper cell diversity and plasticity. *Trends in immunology*, 34(5):200–7, may 2013.

[69] Shimon Sakaguchi, Kajsa Wing, and Makoto Miyara. Regulatory T cells - A brief history and perspective. *European Journal of Immunology*, 37(SUPPL. 1):S116–S123, nov 2007.

[70] S Sakaguchi, N Sakaguchi, M Asano, M Itoh, and M Toda. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of immunology (Baltimore, Md. : 1950)*, 155(3):1151–64, aug 1995.

[71] Howard L Weiner, Andre Pires da Cunha, Francisco Quintana, and Henry Wu. Oral tolerance. *Immunological reviews*, 241(1):241–59, may 2011.

[72] Maria Grazia Roncarolo, Silvia Gregori, Manuela Battaglia, Rosa Bacchetta, Katharina Fleischhauer, and Megan K Levings. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews*, 212:28–50, aug 2006.

[73] J Geginat, M Paroni, F Facciotti, P Gruarin, I Kastirr, F Caprioli, M Pagani, and S. Abrignani. The CD4-centered universe of human T cell subsets. *Seminars in Immunology*, 25(4):252–262, nov 2013.

[74] Jacob D Estes, Stephen Wietgrefe, Timothy Schacker, Peter Southern, Greg Beilman, Cavan Reilly, Jeffrey M Milush, Jeffrey D Lifson, Donald L Sodora, John V Carlis, and Ashley T Haase. Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor beta 1-positive regulatory T cells and begins in early infection. *The Journal of infectious diseases*, 195(4):551–61, feb 2007.

[75] T Aarvak, M Chabaud, P Miossec, and J B Natvig. IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *Journal of immunology (Baltimore, Md. : 1950)*, 162(3):1246–51, feb 1999.

[76] Peter K K Wong, Paul J. Egan, Ben A. Croker, Kristy O'Donnell, Natalie A. Sims, Sarah Drake, Hiu Kiu, Edward J McManus, Warren S. Alexander, Andrew W. Roberts, and Ian P. Wicks. SOCS-3 negatively regulates innate and adaptive immune mechanisms in acute IL-1-dependent inflammatory arthritis. *Journal of Clinical Investigation*, 116(6):1571–1581, jun 2006.

[77] Kensuke Takada and Stephen C. Jameson. Naive T cell homeostasis: from awareness of space to a sense of place. *Nature reviews. Immunology*, 9(12):823–32, dec 2009.

[78] Kenneth M Murphy and Steven L Reiner. The lineage decisions of helper T cells. *Nature reviews. Immunology*, 2(12):933–44, dec 2002.

[79] Natascha Hermann-Kleiter and Gottfried Baier. Review article NFAT pulls the strings during CD4+ T helper cell effector functions. *Blood*, 115(15):2989–2997, 2010.

[80] Grusby M J Glimcher L H Kim JI Ho IC, J I Kim, I C Ho, M J Grusby, and L H Glimcher. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity*, 10(6):745–751, jun 1999.

[81] S. Q. Crome, A. Y. Wang, and M. K. Levings. Translational mini-review series on Th17 cells: Function and regulation of human T helper 17 cells in health and disease, feb 2010.

[82] Maria Ciofani, Aviv Madar, Carolina Galan, Maclean Sellars, Kieran Mace, Florencia Pauli, Ashish Agarwal, Wendy Huang, Christopher N Parkurst, Michael Muratet, Kim M Newberry, Sarah Meadows, Alex Greenfield, Yi Yang, Preti Jain, Francis K Kirigin, Carmen Birchmeier, Erwin F Wagner, Kenneth M Murphy, Richard M Myers, Richard Bonneau, and Dan R Littman. A validated regulatory network for Th17 cell specification. *Cell*, 151(2):289–303, oct 2012.

[83] Mio Kawaguchi, Mitsuru Adachi, Naruhito Oda, Fumio Kokubu, and Shau Ku Huang. IL-17 cytokine family, 2004.

[84] John E Sims and Dirk E Smith. The IL-1 family: regulators of immunity. *Nature reviews. Immunology*, 10(2):89–102, feb 2010.

[85] Mikako Maruya1 Keiichiro Suzuki Yasuko Doi Yumi Tsutsui Lucia M. Kato Sidonia Fagarasan Shimpei Kawamoto, Thinh H. Tran. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science (New York, N.Y.)*, 336(6080):485–9, 2012.

[86] Shane Crotty, Robert J Johnston, and Stephen P Schoenberger. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nature Immunology*, 11(2):114–120, 2010.

[87] Angelina M Bilate and Juan J Lafaille. Induced CD4(+)Foxp3(+) Regulatory T Cells in Immune Tolerance. *Annual review of immunology*, 30:733–58, mar 2011.

[88] Jochen Huehn, Julia K Polansky, and Alf Hamann. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nature reviews. Immunology*, 9(2):83–89, feb 2009.

[89] Oscar Palomares, Görkem Yaman, Ahmet K. Azkur, Tunc Akkoc, Mübeccel Akdis, and Cezmi A. Akdis. Role of Treg in immune regulation of allergic diseases. *European journal of immunology*, 40(5):1232–40, may 2010.

[90] Liliana Attisano and Jeffrey L. Wrana. Signal transduction by the TGF-beta superfamily. *Science (New York, N.Y.)*, 296(5573):1646–1647, 2002.

[91] Eun Sook Hwang, Susanne J Szabo, Pamela L Schwartzberg, Laurie H Glimcher, and Schwartzberg P L Glimcher L H Hwang ES Szabo SJ. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science*, 307(5708):430–433, jan 2005.

[92] Jinfang Zhu, Hidehiro Yamane, We William E WE Paul, and Zhu J Yamane H Paul WE. Differentiation of effector CD4 T cell populations. *Annual review of immunology*, 28(1):445–89, jan 2010.

[93] B. Afzali, P. Mitchell, R. I. Lechler, S. John, and G. Lombardi. Translational mini-review series on Th17 cells: Induction of interleukin-17 production by regulatory T cells, feb 2010.

[94] E de Jong, T Suddason, and G M Lord. Translational mini-review series on Th17 cells: development of mouse and human T helper 17 cells. *Clinical and experimental immunology*, 159(2):148–58, mar 2010.

[95] Cooney RN. Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. *Shock*, 17:83–90, 2002.

[96] Noriko Komatsu, Maria Encarnita Mariotti-Ferrandiz, Ying Wang, Bernard Malissen, Herman Waldmann, and Shohei Hori. Heterogeneity of natural Foxp3+ T cells: a committed regulatory

T-cell lineage and an uncommitted minor population retaining plasticity. *Proceedings of the National Academy of Sciences of the United States of America*, 106(6):1903–1908, feb 2009.

[97] Michael Peine, Sebastian Rausch, Caroline Helmstetter, Anja Fröhlich, Ahmed N. Hegazy, Anja A. Kühl, Christoph G. Grevelding, Thomas Höfer, Susanne Hartmann, and Max Löhning. Stable T-bet+GATA-3+ Th1/Th2 Hybrid Cells Arise In Vivo, Can Develop Directly from Naive Precursors, and Limit Immunopathologic Inflammation. *PLoS Biology*, 11(8):e1001633, aug 2013.

[98] Guangpu Shi, Catherine A. Cox, Barbara P. Vistica, Eric F. Wawrousek, Igal Gery, Cuiyan Tan, Eric F. Wawrousek, and Igal Gery. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *Journal of immunology (Baltimore, Md. : 1950)*, 181(10):7205–13, nov 2008.

[99] Ivaylo I Ivanov, Koji Atarashi, Nicolas Manel, Eoin L Brodie, Tatsuichiro Shima, Ulas Karaoz, Dongguang Wei, Katherine C Goldfarb, Clark a Santee, Susan V Lynch, Takeshi Tanoue, Akemi Imaoka, Kikuji Itoh, Kiyoshi Takeda, Yoshinori Umesaki, Kenya Honda, and Dan R Littman. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*, 139(3):485–98, oct 2009.

[100] Elisabetta Volpe, Maxime Touzot, Nicolas Servant, Marie-Annick Annick Marloie-Provost, Philippe Hupé, Emmanuel Barillot, and Vassili Soumelis. Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production. *Blood*, 114(17):3610–4, oct 2009.

[101] Sascha Rutz, Céline Eidenschenk, and Wenjun Ouyang. IL-22, not simply a Th17 cytokine. *Immunological Reviews*, 252(1):116–132, 2013.

[102] Marc Veldhoen, Catherine Uyttenhove, Jacques van Snick, Helena Helmby, Astrid Westendorf, Jan Buer, Bruno Martin, Christoph Wilhelm, Brigitta Stockinger, and Veldhoen M Uyttenhove C van Snick J Helmby H Westendorf A et al. Transforming growth factor-beta 'reprograms'the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature immunology*, 9(12):1341–6, dec 2008.

[103] Dardalhon V Awasthi A Kwon H Galileos G Gao W Et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol*, 9:1347–1355, 2008.

[104] Roza I Nurieva, Yeonseok Chung, Daehee Hwang, Xuexian O Yang, Hong Soon Kang, Li Ma, Yi-hong Wang, Stephanie S Watowich, Anton M Jetten, Qiang Tian, Chen Dong, Hwang D Yang X O Kang H S et al Nurieva RI Chung Y, Hwang D Yang X O Kang H S et al Nurieva RI Chung Y, and Nurieva R I Chung Y Hwang D Yang X O Kang H S Et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity*, 29(1):138–49, jul 2008.

[105] Thomas Duhen, Rebekka Duhen, Antonio Lanzavecchia, Federica Sallusto, and Daniel J. Campbell. Functionally distinct subsets of human FOXP3 + Treg cells that phenotypically mirror effector Th cells. *Blood*, 119(19):4430–4440, 2012.

[106] Meghan a Koch, Glady's Tucker-Heard, Nikole R Perdue, Justin R Killebrew, Kevin B Urdahl, Daniel J Campbell, Koch M A Tucker Heard G Perdue N R Killebrew J R Urdahl K B Et al, and Perdue N R Killebrew J R Urdahl K B Campbell D J Koch MA Tucker-Heard G. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nature immunology*, 10(6):595–602, jun 2009.

[107] Elizabeth A Wohlfert, John R Grainger, Nicolas Bouladoux, Joanne E Konkel, Guillaume Oldenhove, Carolina Hager Ribeiro, Jason A Hall, Ryoji Yagi, Shruti Naik, Ravikiran Bhairavabhotla, William E Paul, Remy Bosselut, Gang Wei, Keji Zhao, Mohamed Oukka, Jinfang Zhu, and Yasmine Belkaid. GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. *The Journal of clinical investigation*, 121(11):4503–4515, nov 2011.

[108] Fabiola Osorio, Salomé LeibundGut-Landmann, Matthias Lochner, Katharina Lahl, Tim Sparwasser, Gérard Eberl, and Caetano Reis e Sousa. DC activated via dectin-1 convert Treg into IL-17 producers. *European journal of immunology*, 38(12):3274–3281, dec 2008.

[109] Sagar P. Bapat, Jae Myoung Suh, Sungsoon Fang, Sihao Liu, Yang Zhang, Albert Cheng, Carmen Zhou, Yuqiong Liang, Mathias LeBlanc, Christopher Liddle, Annette R. Atkins, Ruth T. Yu, Michael Downes, Ronald M. Evans, and Ye Zheng. Depletion of fat-resident Treg cells prevents age-associated insulin resistance. *Nature*, 2015.

[110] Jeffrey Deiuliis, Zubair Shah, Nilay Shah, Bradley Needleman, Dean Mikami, Vimal Narula, Kyle Perry, Jeffrey Hazey, Thomas Kampfrath, Madhukar Kollengode, Qinghua Sun, Abhay R Satoskar, Carey Lumeng, Susan Moffatt-Bruce, and Sanjay Rajagopalan. Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers. *PloS one*, 6(1):e16376, 2011.

[111] Jorg Van Loosdregt, Yvonne Vercoulen, Teun Guichelaar, Yoony Y J Gent, Jeffrey M. Beekman, Olivier Van Beekum, Arjan B. Brenkman, Dirk Jan Hijnen, Tuna Mutis, Eric Kalkhoven, Berent J Prakken, and Paul J. Coffer. Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood*, 115(5):965–974, feb 2010.

[112] Penelope A. Morel Natasa Miskov-Zivanov, Michael S. Turner, Lawrence P. Kane, Faeder, James R., Natasa Miskov-Zivanov, Michael S. Turner, Lawrence P. Kane, Penelope a. Morel, James R Faeder, James R Faeder November, and James R Faeder. The Duration of T Cell Stimulation Is a Critical Determinant of Cell Fate and Plasticity. *Science Signaling*, 6(300):ra97–ra97, nov 2013.

[113] Angelina M Bilate and Juan J Lafaille. Induced CD4 + Foxp3 + Regulatory T Cells in Immune Tolerance. *Annual Review of Immunology*, 30(1):733–758, jan 2012.

[114] Mandy J McGeachy, Kristian S Bak-Jensen, Yi Chen, Cristina M Tato, Wendy Blumenschein, Terrill McClanahan, and Daniel J Cua. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nature immunology*, 8(12):1390–7, dec 2007.

[115] Kwon H Galileos G Gao W et al Dardalhon V Awasthi A and Kwon H Galileos G Gao W et al Dardalhon V Awasthi A. IL-4 inhibits TGF-β-induced Foxp3+ T cells, and together with TGF-β, generates IL-9+IL-10+ Foxp3- effector T cells. *Nat Immunol*, 9:1347–1355, 2008.

[116] Mandy J McGeachy, Kristian S Bak-Jensen, Y I Chen, Cristina M Tato, Wendy Blumenschein, Terrill McClanahan, and Daniel J Cua. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell–mediated pathology. *Nature immunology*, 8(12):1390–1397, 2007.

[117] H Jonuleit, E Schmitt, G Schuler, J Knop, and A H Enk. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *The Journal of experimental medicine*, 192(9):1213–1222, nov 2000.

[118] Sascha Rutz and Wenjun Ouyang. Regulation of interleukin-10 and interleukin-22 expression in T helper cells. *Current opinion in immunology*, 23(5):605–12, oct 2011.

[119] a W Butch, G H Chung, J W Hoffmann, and M H Nahm. Cytokine expression by germinal center cells., 1993.

[120] Victor L Perez, James A Lederer, Andrew H Lichtman, and Abul K Abbas. Stability of Th1 and Th2 populations. *International Immunology*, 7(5):869–875, may 1995.

[121] Marc Panzer, Selina Sitte, Stefanie Wirth, Ingo Drexler, Tim Sparwasser, and David Voehringer. Rapid in vivo conversion of effector T cells into Th2 cells during helminth infection. *Journal of Immunology*, 188(2):615–23, jan 2012.

[122] Ahmed N. Hegazy, Michael Peine, Caroline Helmstetter, Isabel Panse, Anja Fr??hlich, Andreas Bergthaler, Lukas Flatz, Daniel D. Pinschewer, Andreas Radbruch, and Max L??hning. Interferons Direct Th2 Cell Reprogramming to Generate a Stable GATA-3+T-bet+ Cell Subset with Combined Th2 and Th1 Cell Functions. *Immunity*, 32(1):116–128, 2010.

[123] Yun Kyung Lee, Henrietta Turner, Craig L Maynard, James R Oliver, Dongquan Chen, Charles O Elson, Casey T Weaver, Maynard C L Oliver J R Chen D et al Lee YK Turner H, and Maynard C L Oliver J R Chen D et al Lee YK Turner H. Late developmental plasticity in the T helper 17 lineage. *Immunity*, 30(1):92–107, jan 2009.

[124] David Bending, Hugo De la Peña, Marc Veldhoen, Jenny M Phillips, Catherine Uyttenhove, Brigitta Stockinger, and Anne Cooke. Highly purified Th17 cells from BDC2.5NOD mice

convert into Th1-like cells in NOD/SCID recipient mice. *The Journal of clinical investigation*, 119(3):565–72, mar 2009.

[125] Keiji Hirota, João H Duarte, Marc Veldhoen, Eve Hornsby, Ying Li, Daniel J Cua, Helena Ahlfors, Christoph Wilhelm, Mauro Tolaini, Ursula Menzel, Anna Garefalaki, Alexandre J Potocnik, and Brigitta Stockinger. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nature immunology*, 12(3):255–63, mar 2011.

[126] Estelle Bettelli, Yijun Carrier, Wenda Gao, Thomas Korn, Terry B. Strom, Mohamed Oukka, Howard L. Weiner, and Vijay K. Kuchroo. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*, 441(7090):235–238, may 2006.

[127] Xuyu Zhou, Lukas T Jeker, Brian T Fife, Shirley Zhu, Mark S Anderson, Michael T McManus, and Jeffrey a Bluestone. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *The Journal of experimental medicine*, 205(9):1983–91, sep 2008.

[128] Nicola Gagliani, Maria Carolina Amezcua Vesely, Andrea Iseppon, Leonie Brockmann, Hao Xu, Noah W. Palm, Marcel R. de Zoete, Paula Licona-Limón, Ricardo S. Paiva, Travers Ching, Casey Weaver, Xiaoyuan Zi, Xinghua Pan, Rong Fan, Lana X. Garmire, Matthew J. Cotton, Yotam Drier, Bradley Bernstein, Jens Geginat, Brigitta Stockinger, Enric Esplugues, Samuel Huber, and Richard A. Flavell. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature*, 523(7559):221–5, apr 2015.

[129] Thomas Duhen, Rebekka Geiger, David Jarrossay, Antonio Lanzavecchia, and Federica Sallusto. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature immunology*, 10(8):857–863, aug 2009.

[130] Linyuan Lü and Tao Zhou. Link prediction in complex networks: A survey. *Physica A: Statistical Mechanics and its Applications*, 390(6):1150–1170, mar 2011.

[131] Youn Soo Choi, Jessica a Yang, and Shane Crotty. Dynamic regulation of Bcl6 in follicular helper CD4 T (Tfh) cells. *Current opinion in immunology*, 25(3):366–72, jun 2013.

[132] Keiji Hirota, João H Duarte, Marc Veldhoen, Eve Hornsby, Ying Li, Daniel J Cua, Helena Ahlfors, Christoph Wilhelm, Mauro Tolaini, Ursula Menzel, Anna Garefalaki, Alexandre J Potocnik, and Brigitta Stockinger. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nature immunology*, 12(3):255–63, mar 2011.

[133] Masayuki Tsuji, Noriko Komatsu, Shimpei Kawamoto, Keiichiro Suzuki, Osami Kanagawa, Tasuku Honjo, Shohei Hori, and Sidonia Fagarasan. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science (New York, N.Y.)*, 323(5920):1488–92, mar 2009.

[134] MadhavD Sharma, Lei Huang, Jeong Hyeon Choi, Eun Joon Lee, JamesM Wilson, Henrique Lemos, Fan Pan, BruceR Blazar, DrewM Pardoll, AndrewL Mellor, Huidong Shi, and DavidH Munn. An Inherently Bifunctional Subset of Foxp3+ T Helper Cells Is Controlled by the Transcription Factor Eos. *Immunity*, 38(5):998–1012, may 2013.

[135] Arian Laurence, Shoba Amarnath, Jacopo Mariotti, Yong Chan Kim, Jason Foley, Michael Eckhaus, John J O'Shea, and Daniel H Fowler. STAT3 transcription factor promotes instability of nTreg cells and limits generation of iTreg cells during acute murine graft-versus-host disease. *Immunity*, 37(2):209–222, aug 2012.

[136] Jason S Stumhofer, Jonathan S Silver, Arian Laurence, Paige M Porrett, Tajie H Harris, Laurence A Turka, Matthias Ernst, Christiaan J M Saris, John J O'Shea, and Christopher A Hunter. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature immunology*, 8(12):1363–1371, dec 2007.

[137] Lionel Apetoh, Francisco J Quintana, Caroline Pot, Nicole Joller, Sheng Xiao, Deepak Kumar, Evan J Burns, David H Sherr, Howard L Weiner, and Vijay K Kuchroo. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nature immunology*, 11(9):854–861, sep 2010.

[138] Kastelein R. Hunter CA1. Interleukin-27: balancing protective and pathological immunity. *Immunity*, 37(6):960–9, dec 2012.

[139] Max Löhning, Ahmed N. Hegazy, Daniel D. Pinschewer, Dorothea Busse, Karl S. Lang, Thomas Höfer, Andreas Radbruch, Rolf M. Zinkernagel, Hans Hengartner, M. Lohning, Ahmed N. Hegazy, Daniel D. Pinschewer, Dorothea Busse, Karl S. Lang, T. Hofer, Andreas Radbruch, Rolf M. Zinkernagel, and Hans Hengartner. Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *The Journal of experimental medicine*, 205(1):53–61, jan 2008.

[140] C Neumann, Frederik Heinrich, Katrin Neumann, Victoria Junghans, M F Mashreghi, Jonas Ahlers, Marko Janke, Christine Rudolph, Nadine Mockel-Tenbrinck, Anja A. Kühl, Markus M. Heimesaat, Charlotte Esser, S H Im, Andreas Radbruch, Sascha Rutz, and Alexander Scheffold. Role of Blimp-1 in programing Th effector cells into IL-10 producers. *The Journal of experimental medicine*, 211(9):1807–1819, aug 2014.

[141] Enric Esplugues, Samuel Huber, Nicola Gagliani, Anja E. Hauser, Terrence Town, Yisong Y. Wan, William O'Connor, Anthony Rongvaux, Nico Van Rooijen, Ann M. Haberman, Yoichiro Iwakura, Vijay K. Kuchroo, Jay K. Kolls, Jeffrey A. Bluestone, Kevan C. Herold, and Richard A. Flavell. Control of TH17 cells occurs in the small intestine. *Nature*, 475(7357):514–8, jul 2011.

[142] Christina Heinemann, Sylvia Heink, Franziska Petermann, Ajithkumar Vasanthakumar, Veit Rothhammer, Elien Doorduijn, Meike Mitsdoerffer, Christopher Sie, Olivia Prazeres da Costa, Thorsten Buch, Bernhard Hemmer, Mohamed Oukka, Axel Kallies, and Thomas Korn. IL-27 and IL-12 oppose pro-inflammatory IL-23 in CD4+ T cells by inducing Blimp1. *Nature communications*, 5(May):3770, may 2014.

[143] Kathryn E Wellen and Gökhan S Hotamisligil. Inflammation, stress, and diabetes. *The Journal of clinical investigation*, 115(5):1111–9, may 2005.

[144] Satoshi Nishimura, Ichiro Manabe, Mika Nagasaki, Koji Eto, Hiroshi Yamashita, Mitsuru Ohsugi, Makoto Otsu, Kazuo Hara, Kohjiro Ueki, Seiryo Sugiura, Kotaro Yoshimura, Takashi Kadowaki, and Ryozo Nagai. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nature medicine*, 15(8):914–20, aug 2009.

[145] Jonathan N Glickman, Jian Liu, Adeline Divoux, Jiusong Sun, Jie Zhang, Karine Cle, Galina K Sukhova, Paul J Wolters, Juan Du, Cem Z Gorgun, Alessandro Doria, Peter Libby, S Hotamisligil, Guo-Ping Shi, Richard S Blumberg, Barbara B Kahn, Karine Clément, Jonathan N Glickman, Galina K Sukhova, Paul J Wolters, Juan Du, Cem Z Gorgun, Alessandro Doria, Peter Libby, Richard S Blumberg, Barbara B Kahn, Gökhan S Hotamisligil, and Guo-Ping Shi. Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nature medicine*, 15(8):940–5, aug 2009.

[146] Carlos Gershenson. Guiding the Self-organization of Random Boolean Networks. *Arxiv preprint arXiv:1005.5733*, 2010.

[147] M Aldana. Boolean dynamics of networks with scale-free topology. *Physica D: Nonlinear Phenomena*, 185(1):45–66, oct 2003.

[148] Enrique Balleza, Elena R Alvarez-Buylla, Alvaro Chaos, Stuart Kauffman, Ilya Shmulevich, and Maximino Aldana. Critical dynamics in genetic regulatory networks: examples from four kingdoms. *PloS one*, 3(6):e2456, jan 2008.

[149] L. Mendoza, D. Thieffry, and E. R. Alvarez-Buylla. Genetic control of flower morphogenesis in Arabidopsis thaliana: a logical analysis. *Bioinformatics (Oxford, England)*, 15(7-8):593–606, jul 1999.

[150] Réka Reká Albert, Albert Laszlo Barabasi, Albert-László Barabási, and Notre Dame. Statistical mechanics of complex networks. *Review of Modern Physics*, 74(January):47–97, 2002.

[151] Hidde de Jong, Claudine Chaouiya, Denis Thieffry, Luis Mendoza, Hidde de Jong, Claudine Chaouiya, Denis Thieffry, Mendoza L, Hidde de Jong, and Luis Mendoza. A network model for the control of the differentiation process in Th cells. *Biosystems*, 84(2):101–114, may 2006.

[152] Luis Mendoza and Fátima Pardo. A robust model to describe the differentiation of T-helper cells. *Theory in biosciences = Theorie in den Biowissenschaften*, pages 283–293, oct 2010.

[153] Aurélien Naldi, Jorge Carneiro, Claudine Chaouiya, and Denis Thieffry. Diversity and Plasticity of Th Cell Types Predicted from Regulatory Network Modelling. *PLoS Computational Biology*, 6(9):e1000912, sep 2010.

[154] Aurélien Naldi, Pedro T Monteiro, Christoph Müssel, Hans A Kestler, Denis Thieffry, Ioannis Xenarios, Julio Saez-Rodriguez, Tomas Helikar, Claudine Chaouiya, Reka Albert, Matteo Barberis, Laurence Calzone, Anastasia Chasapi, Thomas Cokelaer, Isaac Crespo, Julien Dorier, Andreas Dräger, Céline Hernandez, Michael Hucka, Hidde De Jong, Sarah M. Keating, Steffen Klamt, Hannes Klarner, Reinhard Laubenbacher, Nicolas Le Novère, Anne Niknejad, Nicolas Rodriguez, Heike Siebert, Gautier Stoll, and Jorge G T Zañudo. Cooperative development of logical modelling standards and tools with CoLoMoTo. *Bioinformatics*, 31(7):1154–1159, 2015.

[155] Luis Mendoza. A Virtual Culture of CD4+ T Lymphocytes. *Bulletin of mathematical biology*, jan 2013.

[156] Adria Carbo, Raquel Hontecillas, Barbara Kronsteiner, Monica Viladomiu, Mireia Pedragosa, Pinyi Lu, Casandra W Philipson, Stefan Hoops, Madhav Marathe, Stephen Eubank, Keith Bisset, Katherine Wendelsdorf, Abdul Jarrah, Yongguo Mei, and Josep Bassaganya-Riera. Systems modeling of molecular mechanisms controlling cytokine-driven CD4+ T cell differentiation and phenotype plasticity. *PLoS computational biology*, 9(4):e1003027, apr 2013.

[157] Pablo Martínez-Sosa and Luis Mendoza. The regulatory network that controls the differentiation of T lymphocytes. *Bio Systems*, 113(2):96–103, aug 2013.

[158] Luis Mendoza and Akram Méndez. A dynamical model of the regulatory network controlling lymphopoiesis. *BioSystems*, 137:26–33, 2015.