A Quantitative Approach to Dominant Tolerance

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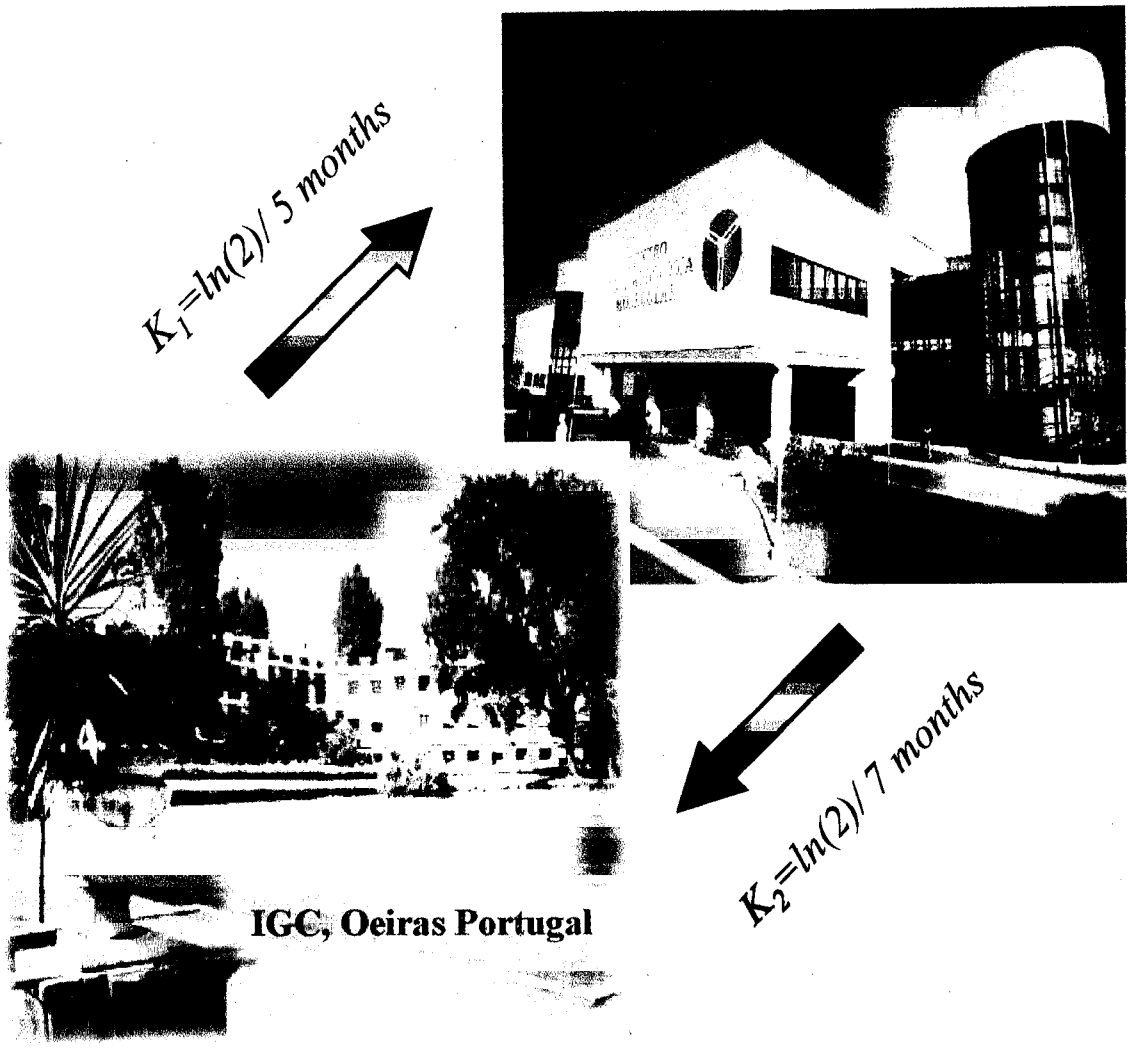

University of Porto
Portugal, 2002

A QUANTITATIVE APPROACH TO DOMINANT TOLERANCE

Kalet León Monzón

Dissertação de candidatura ao grau de doctour
Apresentada ao Instituto de Ciências Biomédicas Abel Salazar,
Universidade do Porto 2002.

In Portugal

Supervisor
Dr. Jorge Carneiro
Gulbenkian Institute of Science

Co-Supervisor:
Prof. Dr. Manuel Vilanova,
Institute of Biomedical Sciences, Abel Salazar

In Cuba

Supervisor
Prof. Dr. Agustín Lage
Center of Molecular Immunology

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To my wife and my daughter

Thanks for be the light of these days

ACKNOWLEDGMENTS (AGRADECIMIENTOS)

I would like to thank:

To Dr. Jorge Carneiro for being my supervisor, my mentor and my friend all at once. I still remember the first time we meet in Havana back to April 1998. Walking around the CIM you proposed me to be your PhD student, not without quoting your lack of experience and youngness. It's been a long way gone since then, including many good, many funny and some not too good moments. It has been a great experience to work next to you, under your supervision, in the Theoretical Immunology Group at the IGC. Thanks for the many useful, interesting and enlightening scientific discussions. Thanks for the always-unconditional support and thanks overall for being my friend.

To Professor Antonio Coutinho, for all the institutional support, which as Director of the Gulbenkian Institute of science, you gave to my work and to the cooperation between the IGC and the Center of Molecular Immunology. Thanks also for being a great immunologist, which has clearly influenced my personal view of the Immune System.

To the late Prof. Dr. Mario Arala Chaves for kindly accepting to be the original co-supervisor of this project and to Prof. Dr. Manuel Vilanova for kindly taking over this responsibility.

To my unconditional roommate in the theoretical wing of the IGC, João Sousa. Thanks for sharing with me many of the experience along this "PhD game". Thanks for the many advices in computational matters and for introducing me to "Mathematica". Thanks for helping me to have a nicer time over here.

To my friend and colleague at the theoretical immunology group at the IGC Dr. Jose Faro. I still don't know how you managed to stand me for almost 2 years (on splits periods) of sharing apartments, always near Palmeiras (jejeje). Thanks for the many interesting, some times funny and some times estrange scientific and non-scientific discussions. Thanks for turning my life easier and nicer over here.

To my colleagues of Jocelyne's Lab (Jocelyne, Tiago, Iris, Vanessa, Shohei, Mannel, Deborah). Thanks for patiently resisting my sometimes long and "full of mathematics" theoretical

dissertations on dominant tolerance. Thanks for being the experimental counterpart of this work and for contributing to create a FORUM for discussing dominant tolerance at the IGC, from which this Thesis definitely profits.

To all the very nice and friendly people that I've met and shared with along these 4 years around the IGC. It is a long list, which I feel unable to reproduce down here without excluding someone. Thanks for being around, for the barbecues, restaurants, dinners, beers and son on. You did a great contribution to the development of this thesis, by helping me to feel happy far from my home and my family.

This was all for the English part. I'm sorry but there are some things, which need to be said in Spanish.

Me gustaría agradecer:

A los Doctores Agustín Lage y Rolando Pérez por la confianza que siempre han depositado en mí y de la que espero ser siempre un digno merecedor. Gracias por el apoyo y el aliento que siempre le han brindado a mi trabajo. Gracias también por siempre forzarme a ir un poquito más allá y enseñarme que cada oportunidad es también un reto y un compromiso. Gracias por las múltiples discusiones científicas, que a lo largo de estos años hemos sostenido. Ellas contribuyeron decisivamente a mi necesaria biologización y a la conformación del pensamiento inmunológico que hoy se expone en esta tesis. Gracias espercialmente al Dr. Agustín Lage por la supervisión directa de esta tesis.

A la pequeña, pero creciente pandilla de los modeladores del CIM. Gracias por crear un magnífico ambiente de trabajo en nuestro pequeño local de investigaciones. Las largas jornadas de esta tesis no hubieran sido lo mismo sin ustedes. Gracias especialmente a Ernesto, por haber sido desde el primer día para mí el buen jefe y el amigo.

A mis compañeros de la Dirección de Investigaciones del Centro de Inmunología Molecular (CIM). A la vieja y ya no tan vieja guardia (Rolando, Lage, Luis Enrique, Maruchi, Juan Félix, Valiente, Irene, Cristina, Normando, Adolfo, Ernesto, Eduardito, Belinda, Montero, Amparo, Gisela, Mauro, Fifi, Adriana Car). A ustedes les agradezco ante todo el haberme dado el privilegio de integrarme a su colectivo, brindándome su amistad y apoyo. Gracias por haber creado en el CIM, con su

esfuerzo cotidiano, una sólida base científica en la que jóvenes como yo pueden apoyarse en busca de nuevos horizontes. A mis contemporáneos (Alexis, Circe, Anita, Ailette, Zaima, Joel) y los más jóvenes (Anabel, Alejandro, Lourdes, Tais, Alain, Ariel, Liván, Ruby, Muste). Gracias simplemente por estar ahí y por preguntar como iba el trabajo y la tesis. Gracias por esa alegría contagiosa y cotidiana que siempre nos ayuda a ser mejores.

A los compañeros de Servisex (Erleys, José Luis y Francisco). Gracias por el trato siempre amable y los muchos cabos que me tiraron. Sin su ayuda hubiese sido difícil seguir el esquema de viajes frequentes al extranjero que implicó esta tesis.

A mis suegros (Marylin y Eduardo) y mi cuñados Milvia y Danielito. Gracias por su apoyo y por haber tomado cuenta de Mayrel y Camila en los largos períodos de ausencia que implicó este trabajo. Saber que estaban con ustedes me dió siempre fuerzas para seguir adelante.

A mis padres, mi hermano, mi tía, mis abuelos. Gracias por haber estado siempre allí y ser ese necesario y sólido retiro espiritual, donde reponemos fuerzas para volver siempre con mayor ímpetu a un nuevo día de trabajo.

A mi esposa (Mayrel) y mi hija (Camila). Gracias por ser la luz de mis días.

DECLARAÇÃO.

Os resultados do trabalho incluídos nesta Dissertação fazem parte de artigos científicos indicados abaixo, alguns dos quais já publicados em revistas internacionais.

No cumprimento do disposto no nº 2 do artigo 8º do Decreto Lei nº 388/70, o autor desta Dissertação declara que participou na concepção e na execução do trabalho que esteve na base desses artigos, bem como na interpretação dos resultados e na redação dos respectivos manuscritos.

TRABALHOS PUBLICADOS:

Leon, K., Perez, R., Lage, A. & Carneiro, J. (2000). Modelling T-cell-Mediated Suppression Dependent on Interactions in Multicellular Conjugates. *J Theor Biol.* **207**, 231-254.

Leon, K., Perez, R., Lage, A. & Carneiro, J. (2001). Three-cell interactions in T cell-mediated suppression? A mathematical analysis of its quantitative implications. *J Immunol.* **166**, 5356-65.

MANUSCRITOS INÉDITOS:

Oliveira V., **Leon K.**, Caramalho I., Hori S., Haury M., Demengeot J. & Carneiro, J. (2002). Target CD4+CD25- T cells promote the growth of regulatory CD4+CD25+ T cells in vitro. Submitted to publication.

Leon, K., Lage, A. & Carneiro, J. (2002). Tolerance and Immunity in a mathematical model of T cell-mediated suppression . Submitted to publication

Leon, K., Faro, J., Lage, A. & Carneiro, J. (2002). Inverse Correlation for the Incidence of Autoimmunity and Infections predicted by a Mathematical model of T cell mediated suppression. Submitted to publication.

The work included in this Thesis has been developed as collaboration between the Center of Molecular Immunology in Habana, Cuba and the Gulbenkian Institute of Sciences in Oeiras, Portugal. These Institutions and the Program Praxis XXI of the Ministério para Ciência e Tecnologia, Portugal (grant Praxis/P/BIA/10094/1998) provided all the necessary financial support.

ABBREVIATIONS AND NOMENCLATURE

ADCC	*Antibody dependent cell cytotoxicity*
Anti CD(x) MAb	*MAb specific to CD(x)*
APC	*Antigen presenting cell*
BCR	*B cell receptor*
CD(x)	*Cell Differentiation antigen (x)*
CDR(x)	*Complementarity Determination Region (x)*
CTLA-(x)	*Citotoxic T lymphocyte Antigen (x)*
DC	*Dendritic Cell*
E/T cells	*Effector/ Target T cells*
EAE	*Experimental Allergic Encephalomyelitis*
GMSF	*Granulocyte-Macrophage Stimulating Factor*
HA	*Hemaglutinin Antigen of the Influenza Virus*
IBD	*Inflammatory Bowel Disease*
IL-(x)	*Interleukin (x)*
IL-(x)R	*Interleukin-(x) receptor*
iTh	*Immature Helper T cell*
LPS	*LipoPolySaccharide*
MAb	*Monoclonal Antibody*
MHC	*Major Histocompatibility Complex*
NOD	*Non-Obese Diabetic*
ODE	*Ordinary Differential Equation*
R cell	*Regulatory T cell*
Rag	*Recombination associated gene*
SCID	*Severe Combined Immuno Deficient.*
SPF	*Specific Pathogen Free*
T cells	*Effector/ Target T cells*
Tc	*Cytotoxic T cell*
TCR	*T cell receptor*
Th	*Helper T cell*
TNF-(x)	*Tumor necrosis factor (x)*
DNA	*DeoxiriboNucleic Acid*
LDV	*Lactate Dehydrogenase Virus*

NZBNZW	*New Zeeland Black-New Zeeland White*
PCC	*Pigeon Cytochrome C*
MBP	*Myelin Basic Protein*
GVHD	*Graft versus Host Disease*
mRNA	*Messenger RiboNuclei Acid*
GAPDH	*Glyceraldehyde-3-Phosphate Dehydrogenase.*

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Summary

The present Thesis aims to further the understanding of immunological self-tolerance mediated by regulatory T cells. A biomathematical approach is followed in which mathematical models were developed, studied and confronted with experimental observations. Two complementary threads were followed. On the one hand, we studied the details of the mechanism by which regulatory T cells interact with other cells and mediate the suppressive function. Here mathematical modeling was used as a tool to compare and assess the plausibility of alternative hypothetical mechanisms. On the other hand, we studied the implications of T cell mediated tolerance for the organization and operation of the immune system, with special focus on self/non-self discrimination and on the etiology of autoimmune diseases. Here modeling served as a tool to bridge different levels of organization, i.e. to understand the systemic consequences of properties of individual cells.

The main working hypothesis was that regulatory T cells effect linked suppression of their target cells when both cells are conjugated simultaneously with the antigen presenting cells (APC). Multicellular interactions are often found in biological systems but despite the general interest a formalism describing this type of interactions this was not available in the literature. Hence, we developed a simple formalism describing the formation of multicellular conjugates and quantifying the frequency distribution of conjugates with different stoichiometries. This formalism was used to represent interaction terms in population dynamic models of regulatory cells and their targets cells.

We designed four models representing the population dynamics of regulatory and target T cells, which implement alternative mechanisms of linked suppression previously proposed in the literature. We made phase plane and bifurcation analysis of each model, and identified its pros and cons in terms of the relationship with the large body of *in vivo* experimental observations on T cell mediated tolerance. We argued that accounting for the quantitative details of adoptive transfers of tolerance requires models that: (i) possess bistable regimes in which either regulatory or target T cells dominate the steady state interpreted as tolerance and immunity respectively, depending on initial conditions; and (ii) possess a steady state interpreted as tolerance in which both regulatory and target cell populations coexist. We showed that only models in which the growth of regulatory T cells is strictly dependent on their target T cells bear these two properties. To further assess these candidate models, we challenged them by their capacity to explain observations *in vitro*. This analysis allowed us to quantify the efficiency of *in vitro* suppression dependent on multicellular conjugates and to show that it has an upper bound. Comparing this upper bound with the efficiency of suppression *in vitro*, we rejected those models in which suppression is mediated by simple

competition for APCs or in which the regulatory T cell population is unable to grow. Thus, as a whole our theoretical analysis of alternative models allowed to narrow down the number of candidate hypotheses to those in which the population regulatory T cells grows as a function of the target T cells they suppress. The growth of this population may be driven by a target cell-dependent growth factor and/or may result from differentiation of target cells to the regulatory phenotype. We addressed and confirmed the first hypothesis by designing and carrying out new *in vitro* suppression assays in which proliferation of regulatory CD4+CD25+ and target CD4+CD25- T cells could be quantified concomitantly. This study showed for the first time that regulatory CD25+ T cells, which are unable to proliferate by themselves when stimulated *in vitro*, will proliferate when costimulated together with target CD25- T cells. Moreover, the proliferation of regulatory cells is correlated with the expansion of the target cell population suggesting that the two cell types might share a target cell-dependent growth factor, whose production is inhibited by regulatory T cells. The nature of this growth factor is discussed to be most likely IL-2 and it is argued that this would provide a mechanistic rational for the involvement of IL-2, IL-2 receptor and CD4+CD25+ T cells in self tolerance.

Having developed and shown the plausibility of our model for T cell mediated tolerance we explored its implications for the understanding of self/non-self discrimination. An issue of specificity is inescapable when considering a mechanism of tolerance by "linked recognition" of the APC: how can this "non-specific" regulation allow for tolerance to body components and immunity to invading pathogens. To tackle this issue, we simulated the thymic selection and peripheral dynamics of many T cell clones containing regulatory and target cells that interact according to our model. The simulations mimicked the capacity of the immune system to establish robust self-tolerance and reliably mount immune responses to foreign antigens. Mounting an immune response to foreign antigens requires the loss of regulatory cells in APC conjugates, which can happen because ubiquitous self-antigens are displaced from the APCs by foreign antigens and/or because there is an increase in the number of APCs upon the introduction of a foreign antigen. The simulations indicated that realistic balance between tolerance and immunity could only be achieved if the thymus shapes the repertoire so that affinities for self-antigens are upper and lower bounded, but, once this condition is fulfilled, differentiation of thymocytes to regulatory phenotype may be antigen-nonspecific. These results call for a reassessment of the role of positive and negative selection. Positive selection might be necessary to ensure a sufficiently high frequency of autoreactivity in both regulatory and target T cells such that self-tolerance becomes reliable and robust. Negative selection may be required to avoid the emergence at the periphery of very high

affinity anti-self regulatory cells that will make the tolerant state so robust that it could no be broken by the introduction of a foreign antigen.

Finally, we use our model to address the issue of the etiology of autoimmune disorders and its relation to infections. Considering that infection will lead to an increase in APCs and in the presentation of self-antigens we show that acute infections may trigger specific autoimmune diseases but that overall microbial load may protect non-specifically against autoimmunity by reinforcing T cell mediated tolerance. These opposing consequences of infection stem from the non-linearity of the mechanism of interaction between regulatory and target cell populations. Therefore the model solves the otherwise paradox between the hygienist and antigen mimicry hypotheses for the ethiology of autoimmune diseases.

Overall, this Thesis provided the first comprehensive study of different aspects of T cell mediated tolerance by mathematical modeling. We raised and addressed some of the most outstanding problems in the field. We provided some answers and raised many new issues.

Sumário

Esta Dissertação versa o estudo da tolerância ao próprio mediada por células T reguladoras. Seguiu-se uma abordagem biomatemática na qual modelos matemáticos são desenvolvidos, estudados e confrontados com observações experimentais, em duas vertentes. Por um lado, estudamos os detalhes do mecanismo pelo qual as células reguladoras interagem com outras células e medeiam a sua função supressora. Aqui, a modelação matemática foi um instrumento na comparação e avaliação de hipóteses mecanísticas alternativas. Por outro lado, estudamos as implicações da tolerância mediada por células T para a organização e funcionamento do sistema imune, especialmente no que diz respeito a discriminação próprio/estranho e à etiologia das doenças autoimunes. Aqui, a modelação serviu de instrumento para estabelecer uma ponte entre vários níveis de organização, i.e. para compreender as consequências sistémicas de propriedades individuais das células.

A principal hipótese de trabalho foi que a célula T reguladora suprime a sua célula alvo quando ambas se encontram conjugadas simultaneamente com a mesma célula apresentadora de antigénio (APC). Interacções multicelulares são frequentes em sistemas biológicos, no entanto não existia na literatura um formalismo descrevendo este tipo de interacção, apesar do seu interesse geral. Portanto, desenvolvemos um formalismo simples que descreve a formação de conjugados multicelulares e quantifica a distribuição frequências dos conjugados de acordo com a respectiva estequiometria. Este formalismo foi usado para representar os termos de interacção em modelos de dinâmica de populações de células reguladoras e das suas células alvo.

Estabelecemos quatro modelos, que implementam mecanismos alternativos de supressão disponíveis na literatura. Fizemos análise do espaço de fases e bifurcações de cada modelo e identificamos os seus prós e contras em termos da sua relação com o corpo de observações experimentais sobre tolerância mediada por células T *in vivo*. Argumentamos que a capacidade de explicar os detalhes quantitativos das transferências adoptivas de tolerância requer modelos que: (i) possuem um regime de bi-estabilidade em que células reguladoras e células alvo dominam dois estados estacionários interpretados como tolerância e imunidade respectivamente, (ii) possuem um estado estacionário interpretado como tolerância em que as duas populações de células coexistem. Finalmente, mostramos que apenas os modelos em que o crescimento da população reguladora é estritamente dependente das suas células alvo possuem estas duas propriedades. Para melhor avaliar os modelos candidatos, testamos a sua compatibilidade com observações *in vitro*. Esta análise

permitiu-nos quantificar a supressão *in vitro* dependente de conjugados multicelulares, e mostrar que esta tem um limite superior. Comparando este limite superior com os valores experimentais, pudemos rejeitar aqueles modelos em que a supressão é mediada por simples competição pelas APCs ou em que a população reguladora é incapaz de crescer. Portanto, no seu todo, a analise matemática dos modelos alternativos permitiu-nos reduzir significativamente o número de hipóteses, aquelas em que a população de células reguladoras cresce em função da população das suas células alvo. O crescimento da população reguladora pode ser dependente de um factor de crescimento produzido pelas células alvo, e/ou pode resultar da diferenciação de células alvo em células reguladoras. Avaliamos e confirmamos experimentalmente que pelo menos a primeira alternativa é verdadeira. Isto foi feito recorrendo a um ensaio de supressão *in vitro* em que pudemos medir independentemente a proliferação de células T reguladoras CD4+CD25+ e de células alvo CD4+CD25-. Este estudo mostrou pela primeira vez que células T reguladoras CD25+, que são incapazes de proliferar quando estimuladas sozinhas *in vitro*, proliferam significativamente quando estimuladas na presença de células alvo CD25-. Mais ainda, como a proliferação de células reguladoras e células alvo se correlacionou positivamente nestes ensaios, sugerimos que os dois tipos de células partilham o mesmo factor de crescimento, e discutimos a possibilidade deste factor de crescimento ser a IL-2. Argumentamos que esta interpretação permite racionalizar o envolvimento da IL-2, do seu receptor e das próprias células reguladoras CD4+CD25+ na tolerância ao próprio.

Tendo desenvolvido e mostrado a plausibilidade do nosso modelo de tolerância mediada por células T exploramos as suas implicações para a compreensão da discriminação próprio/estranho pelo sistema imunitário. A consideração que o mecanismo de tolerância se processa por "reconhecimento associado" da mesma APC levanta inevitavelmente um problema de especificidade: como é que este sistema relativamente inespecífico de regulação pode permitir tolerância aos componentes do corpo e imunidade contra patogénios estranhos. Para tentar resolver este problema, simulamos a selecção tímica e dinâmica na periferia de múltiplos clones de células T reguladoras e alvo. As simulações reproduzem qualitativamente a capacidade do sistema imune de estabelecer uma tolerância robusta ao próprio e responder eficientemente contra patogénios estranhos. Montar uma resposta imune requer uma perda relativa de células reguladoras por APC, que pode seguir-se à substituição nas APCs dos antigénios ubiquitários do hospedeiro pelos antigénios estranhos e que pode resultar do aumento do número de APCs após o contacto com os antigénios estranhos. As simulações indicaram que um balanço realista entre tolerância e imunidade pode ser obtida se o timo formar o repertório dos receptores das células T de tal forma

que as afinidades para os antigénios do próprio tiverem dois limites superior e inferior, mas desde que esta condição for satisfeita, a diferenciação dos timocitos para o fenotipo regulador pode ser independente do antigénio. Estes resultados sugerem uma reapreciação dos processos de selecção positiva e negativa. A selecção positiva seria necessária para assegurar uma frequência de autoreactividade suficientemente alta por parte das células reguladoras e das células alvo, de modo a que a tolerância ao próprio seja assegurada e robusta. A selecção negativa poderia ser necessária para impedir que a frequência de células reguladoras autoreactivas seja tão grande que tolerância ao próprio seja tão forte que não pode ser quebrada.

Finalmente, usamos o nosso modelo para investigar a etiologia das doenças autoimunes e a sua relação com as infecções. Considerando que uma infecção resulta num incremento do número de APCs e no aumento da apresentação antigénica, mostramos que infecções agudas poderão desencadear doenças autoimunes específicas mas que a carga microbiana a que um hospedeiro está exposto poderão protegê-lo inespecificamente contra patologias autoimunes, por reforço da tolerância mediada por células T. Estas consequências opostas da infecção resultam da não-linearidade do mecanismo de interacção entre populações de células T reguladoras e alvo. Portanto, o modelo propõe uma possível solução para o paradoxo posto pela hipóteses higienista e do mimetismo antigénico sobre a etiologia das doenças autoimunes.

No seu todo, esta Dissertação constitui o primeiro estudo compreensivo dos diferentes aspectos da tolerância mediada por células T por modelação matemática. Levantamos e tentamos resolver alguns dos problema mais relevantes nesta área. Propusemos algumas soluções e levantamos várias outras questões.

Resumé

Cette thèse concerne l'étude de la tolérance du soi par les cellules T régulatrices. Suivant une approche biomathématique, des modèles mathématiques sont developpés, étudiés et confrontés à des observations experimentales, ceci de deux façons complémentaires. Dans la première, nous avons étudié les détails du mécanisme par lequel les céllules régulatrices interagissent avec d'autres céllules et accomplissent leur fonction suppressive. Dans ce domaine, la modélisation mathématique a été un instrument pour la comparation et pour l'évaluation d'hypothèses mécanistiques alternatives. Dans la seconde, nous avons étudié les implications de la tolerance conduite par des céllules T dans l'organisation et le fonctionnement du système immunitaire, particulièrement en ce qui concerne la distinction du soi/non-soi et de l'étiologie des maladies autoimmunes. Ainsi, la modélisation a servi d'instrument pour établir une liaison entre plusieurs niveaux d'organisation ; c'est-à-dire, pour comprendre les conséquences systémiques des propriétés de chaque céllule.

La principale hypothèse de travail est que la céllule T régulatrice supprime sa céllule cible quand elles sont associées simultanément avec la même céllule présentatrice d'antigène (CPA). Des intéractions multicellulaires sont fréquentes dans les systèmes biologiques, néanmoins, il n'existait pas dans la littérature un formalisme simple qui décrive la formation d'agrégats multicellulaires et qui quantifie la distribution des fréquences des agrégats, en accord avec leur respective stechiométrie. Ce formalisme a été utilisé pour représenter les termes d'intéractions entre des populations de céllules régulatrices et de leurs céllules cibles dans des modèles dynamiques.

Nous avons établies quatre modèles qui intègrent les mécanismes alternatifs de suppression proposés dans la littérature. Nous avons réalisé l'analyse de l'espace de phases et de bifurcations de chaque modèle et evalué leurs avantages et désavantages en relation avec toutes les observations experimentales concernant la tolérance accomplie par des céllules T *in vivo*. Nous argumentons que la capacité d'expliquer les détails quantitatifs des transfers adoptifs de tolérance a besoin de modèles qui : (i) possèdent un régime de bi-stabilité où les céllules régulatrices et les céllules cibles dominent deux états stationnaires interprétés respectivement comme tolérance et immunité, (ii) possèdent un état stationnaire interprété comme tolérance où les deux populations de céllules co-existent. Finalement, nous montrons que seuls les modèles où la croissance de la population régulatrice est strictement dépendante de celle des céllules cibles, possèdent ces deux propriétés. Pour mieux évaluer les modèles candidats, nous avons testé leur compatibilité avec des observations *in vitro*. Cette analyse nous a permit de quantifier la suppression *in vitro* dépendante d'agrégat

multicellulaires, et de montrer que celle-ci a une limite supérieure. En comparant cette limite supérieure avec les valeurs expérimentales, nous avons pu rejeter les modèles où la suppression est accomplie par simple compétition pour les CPAs et ceux où la population régulatrice est incapable de s'accroître. Globalement, l'analyse mathématique des modèles alternatifs nous a donc permit de réduir significativement le nombre d'hypothèses et ne retenir que celles dont la population de céllules régulatrices s'accroît en fonction de la population de céllules cibles. La croissance de la population régulatrice peut être dépendante d'un facteur de croissance produit par les céllules cibles, et/ou être la conséquence de différentiation de céllules cibles en céllules régulatrices. Nous avons évalué et confirmé experimentalement que au moins la première alternative est vraie. Ceci a été fait par un test de suppression *in vitro* où nous avons mesuré independamment la prolifération de céllules T régulatrices CD4+CD25+ et celle des céllules cibles CD4+CD25-. Cette étude a montré pour la première fois que les céllules T régulatrices CD25+, qui ne prolifèrent pas quand elles sont stimulées seules *in vitro*, prolifèrent significativement quand elles sont stimulées uniquement en présence de céllules cibles CD25-. De plus, comme la prolifération de céllules régulatrices et de céllules cibles est correlée dans ces expériences, nous sugerons que les deux types de céllules partagent le même facteur de croissance, et nous discutons la possibilité que ce facteur de croissance soit IL-2. Nous argumentons que cette interpretation permet de rationaliser l'enrôlement de l'IL-2, de son récepteur et des céllules régulatrices CD4+CD25+ dans la tolérance au soi.

Ayant développé et démontré la plausibilité de notre modèle de tolérance accomplie par des céllules T, nous avons exploré leurs implications dans la compréhension de la discrimination du soi/non-soi par le système immune. La considération que le mécanisme de tolérance résulte de la « reconnaissance associée» de la même CPA pose inévitablement un problème de spécificité : comment un système relativement non-spécifique de régulation peut permettre à la fois la tolérance aux composants du soi et l'immunité contre les patogènes. Pour essayer de résoudre ce problème, nous avons simulé la sélection thymique et la dynamique dans la périphérie de multiples clones de céllules T régulatrices et cibles. Les simulations reproduisent qualitativement la capacité du système immunitaire d'établir une tolérance robuste au soi et de répondre avec efficacité contre les patogènes. Elaborer une réponse immunitaire nécessite la diminution du nombre de céllules régulatrices par CPA, conséquence de la probable substitution dans les CPAs des antigènes ubiquitaires de l'hôte par les antigènes étrangers. Les simulations ont indiqué qu'un bilan réaliste entre tolérance et immunité peut être obtenu si le thymus forme le répertoire des récepteurs des céllules T d'une telle façon que les affinités pour les antigènes du soi soient limitées. Cependant, une fois cette condition est satisfaite, la différentiation des thymocytes pour un phénotype

régulateur peut être indépendante de l'antigène. Ces résultats sugèrent une réapréciation des processus de sélection positive et négative. La sélection positive serait nécessaire pour assurer une fréquence d'autoréactivité sufisament élevée de la part des céllules régulatrices et des céllules cibles, de façon a ce que la tolérance au soi, soit assurée et robuste. La selection négative pourrait être nécessaire pour empêcher que la fréquence de céllules régulatrices autoreactives soit si élevée que la tolérance au soi ne puissent pas être rompue.

Finalement, nous avons utilisé notre modèle pour étudier l'étiologie des maladies autoimmunes et leur relation avec les infections. En considérant qu'une infection induise une augmentation du nombre de CPAs ainsi qu'une augmentation de la présentation antigénique, nous avons démontré que les infections aigues peuvent induir des maladies autoimmunes spécifiques. Cependant, la charge bactérienne à laquelle un hôte est exposé, pourrait aussi le protéger de façon non spécifique contre des patologies autoimmunes, par renforcement de la tolérance accomplie par les céllules T. Ces conséquences contradictoires à l'infection résultent de la non-linéarité du mécanisme d'interaction entre populations de céllules T régulatrices et cibles. Le modèle propose donc une solution pour le paradoxe proposé par l'hypothèse higièniste et du mimétisme antigénique sur l'étiologie des maladies autoimmunes.

Cette thèse constitue la première étude compréhensive par modélisation mathématique des différents aspects de la tolérance accomplie par des céllules T. Nous avons posé et essayé de résoudre quelques-uns des problèmes les plus critiques dans la matière. Nous avons proposés quelques solutions et nous posons plusieurs questions additionelles.

Chapter 1

INTRODUCTION

1.1 Immunological background

1.1.1 A minimal view of immunology.

The immune system is the set of mechanisms, molecules and cells that mediate the body's resistance to invasion by microorganisms (*i.e.,* immunity). Immunology is the science that studies this body's system of defense. The immune system is typically subdivided in the innate and the adaptive immune components.

The innate immune system is evolutionarily ancient [1], being present almost in every known multi-cellular organism. This system contains both cellular and humoral elements that are able to recognize and react to some chemical structures typically present on infectious microorganisms. In vertebrates several cell types integrate this system, being roughly all the leukocytes that are not lymphocytes, for instance: macrophages, eosinophiles, NK cells and mast cells. Among its humoral components are especially important the molecules of the complement cascade. The recognition capacity of the innate immune system is limited, given the limited set of genetically encoded receptors that mediate its reaction. Classically they have been thought to recognize mainly carbohydrates or Glico-lipids that integrate bacteria's cellular wall [2]. But nowadays with the discovery and study of the toll receptor family [3-5] these recognition capacity has been certainly broader. The receptors of the innate immune system are the result of the evolutionary experience of given species, having been genetically preserved those receptors that confer protection to commonly encounter pathogens.

The adaptive immune system is a much more recent acquisition of evolution [1, 6]. It exists only in vertebrates and it is composed of lymphocytes and their secreted products. This system is characterized by a practically unlimited set of receptors, that are generated by random recombination and mutation of a relatively large set of genes and are expressed under clonal bases by the lymphocytes, i.e. each lymphocyte express only one type of receptor on its membrane. The receptor confers to lymphocytes the capacity to recognize and react to specific aspects of the chemical components of the invader pathogens (antigens). The potential receptor variability is estimated [7, 8] to be around 10^{16}. Adult humans express around 10^{12} different lymphocytes at the given time and 10^{6} new lymphocytes are produced per day in the system. Such enormous recognition capacity together with the seminal studies of Landstainer et al [9], lead immunologists to claim that lymphocyte repertoire is open-ended. Or in other words that for any possible antigen there will be at least one lymphocyte in the repertoire that recognizes it. This property of the immune system is believed to allow it to cope with rapidly mutating pathogens. It also explains the

high specificity of the immunity acquired after reacting with a particular antigen, because the reaction corresponds to the amplification of clones containing the complementary receptors.

Two classes of lymphocytes are clearly distinguished in the immune system. B- lymphocytes recognize regions of the native 3-Dimmensional structure of antigens [10, 11], which are typically proteins or carbohydrates. Upon activation, this B cells proliferate and differentiate into plasma cells that produce and secrete large amounts of antibodies, which are a soluble form of its specific receptor. The antibodies neutralize the pathogenic activity of the invader microorganism and through several potent effector mechanisms promote its destruction. T-lymphocytes recognize the antigen only after being processed and presented by the so-called antigen presenting cells (APC). Classically T cells are known to recognize peptides derived from antigenic proteins [12], once presented in the context of specialized molecules named major histocompatibility complex (MHC-II or MHC-I). But recent data have demonstrated the existence of other non-classical presentations molecules [13-15] that present glicolipids to T cells. T lymphocytes are further classified in cytotoxic (Tc) and helpers (Th) according to their function. Tc once properly activated mediate the lyses of those cells that have been infected by the pathogen. The Th cells are central element in any immune reaction, since once activated they cooperate with both B and Tc cells in mounting the immune responses.

Overall the system appears as a very complex entity, where the innate and adaptive components tightly sinergize in the development of the immune reaction. Even disregarding different specificities of lymphocytes, the system is composed of more than 10 cell types and more than 20 soluble factors that mediate or modulate the activity of these cells. To understand how these elements orchestrate the dynamic of immune response is the challenge of Immunology.

1.1.2 Immunological tolerance and natural tolerance.

At the same level of importance than the concept of immunity and defense to pathogens is the concept of tolerance in immunology. Tolerance is defined as the absence of immune response, which is specific to a given antigen and caused by a previous encounter with the antigen. In other words, if the system does not react to antigen A but it is able to properly react to other antigens, it is said to be tolerant to antigen A. The most relevant example of tolerance is the one to the normal components of the organism, which is typically referred as natural tolerance. The break down of natural tolerance leads to pathogenic immune destruction of normal tissues or, in technical words, to autoimmunity or autoimmune disorders. Understanding natural tolerance is a fundamental and

unresolved problem in Immunology. If the immune system reacts by chemical recognition and there is no difference in the chemical nature of the normal tissue and the infectious pathogens, how does the system effectively prevent the immune reaction to the former while it mounts responses to the later?

Very early in the history of immunology the phenomena of natural tolerance was realized. By the end of the 19-century Ehrlich and Morgenroth (references in [16]) reported the inability to obtain an antibody response to autologous erythrocytes, coining the term *horror autotoxicous* to refer the resistance of the immune system to react and consequently destroy normal body components. Nevertheless, tolerance was not set on the spotlight of immunology until the middle of the 20-century, following Owen pioneer experiments in 1945 [17]. This study showed that despite genetic differences, dyzigotic cattle twins tolerate one another's antigens given that they share the same circulatory system in utero. Sir. Macfarlane Burnet discussed the significance of these results [18, 19], stressing the acquired character of natural tolerance and predicting that tolerance was induced given that immune system was exposed to the antigens in the initial phase of its development. Experimental support for this prediction came later from the experiments of Sir Peter Medawar [20], where mice of a strain A neonatally injected with hematopoietic tissues from the strain B were shown to became tolerant, being unable to reject an adult skin transplantation from the strain B, but not from an unrelated strain C. About the same time tolerance was artificially induced in adult immune system by immunization of excessive amount of antigen leading to the concept of immunological paralysis [21-23]. Since these seminal studies, the mechanisms that mediate tolerance and particularly natural tolerance have been in the core of Immunology and every theoretical construct has faced the question of how the immune system can react to infectious pathogens and keep tolerance to the normal body tissues. A problem metaphorically referred as the self/non-self discrimination problem [24-28].

1.1.3 Theories for self/non-self discrimination.

In this section some of the theories that have been formulated to solve the self/non-self discrimination problem in Immunology are introduced and critically examined. Our objective is to briefly review different theoretical constructs that elaborate on the same theoretical problem addressed in this thesis, introducing at the same time, key elements of immunological knowledge that are required to properly understand this work. For each theory, the theoretical construct is first exposed; the experimental support existent for at least some of the postulates is then presented; and

the positive and negative aspects related to the self/non-self discrimination problem are finally discussed.

1.1.3.1 The Clonal Selection Theory

The Clonal selection theory was formulated by Burnet in 1957 [29] and still nowadays has a strong influence in current immunological thinking. This theory postulates the clonal character of lymphocyte receptors, explaining the immune response as the selective expansion of those clones with receptors specific for a given antigen. To account for natural tolerance this theory proposes that lymphocyte repertoire once generated, is purged from those specificities that recognize normal body tissues (self-antigens) in a process typically referred as central or clonal deletion. For Burnet [30] the lymphocyte repertoire was both generated and purged during embryonic development. Therefore tolerance to body tissues was equated in this theory to the absence of lymphocyte specificities recognizing self-antigens. The discovery that lymphocytes where continuously generated throughout live in normal vertebrate shifted immunological thinking towards a variation of the Burnet theory that was first proposed by Lederberg in 1959 [31]. Lederberg proposed that clonal deletion takes place during an immature state in each individual lymphocyte development. At this immature stage lymphocytes that happen to recognize any available antigen would die. Since body- /self-antigens are always available self-reactivities would be systematically filtered out of the repertoire of mature lymphocytes. Further development of this theory lead to its actual form where selection is believed to take place in specialized organs where lymphocyte development occurs, namely bone marrow for B cells and thymus for T cells [32].

Considerable experimental support exists for this theory on the structural aspect of the clonal expression of lymphocyte receptors [33, 34]. Nowadays, this postulate of clonal selection theory is broadly accepted together with the notion of selection and expansion of specific clones in the course of an immune reaction. Also the existence of clonal deletion induced by specific antigen recognition is well documented. For T cells, clonal deletion is part of a more complex process called thymic selection. In this process, thymocytes are rescued from death if they get an intermediate to low signal through the TCR, but are deleted if the signal is too strong (negative selection) [35-39]. From the total immature T cells produced in the thymus only the 5% are finally exported as mature T cells to periphery, the remaining 95% being lost by apoptosis [40]. Interestingly, negative selection explains only 5% of this massive death [41-44] and the vast majority of the cells die by absence of positive selection (neglect for the specialists). For B cells, the existence of clonal deletion has been also well documented [45-48], although more attention has been pay to T cells in this respect.

Clonal selection theory successfully predicted the clonal character of lymphocytes receptors and provided a simple explanation for the basis of the immune responses that still nowadays prevails in Immunology. Nevertheless in respect to the problem of self/non-self discrimination it is currently regarded as incomplete. Central deletion contributes to natural tolerance but its role is insufficient, and other mechanisms must be called into play. Two major arguments are usually made in this respect. The first argument is that not every self-antigen is expressed in the thymus and bone marrow. The so-called peripheral antigens are then not accessible for negative selection. Although there are counter arguments, namely that APCs can carry the peripheral antigens to the thymus [49] or that many genes encoding peripheral antigens are expressed by medullary epithelial cells in thymus both at the mRNA and protein levels [50]. The second argument against sufficiency of central deletion is that self-reactive T and B lymphocyte are frequently found in the periphery of normal immune system. This fact became evident first for B cells, with the detection self-reactive antibodies [51] in the serum of normal [52] and germ free mice [53]; and second for T cells, with the study of transgenic and double transgenic models where a given antigen is known to be present in the thymus and nevertheless a considerable amount of specific T cells are observed at the periphery [54, 55]. Let's take for instance the double transgenic mouse developed by Lanoue et al [54]. This animal expresses the Hemaglutinin Antigen of the influenza virus (HA) in lymphocytes, and a transgenic TCR receptor specific to HA in the appropriate MHC restriction. This animal contains around 15% of their spleen CD4+ T cells expressing the transgenic TCR, but still is tolerant to the peripheral expression of HA and moreover tolerates further DNA vaccination with HA in a viral vector specific for skeletal muscle [56]. These results clearly show that clonal deletion is incomplete, calling for additional mechanisms to explain natural tolerance.

1.1.3.2 The two-signal model.

Bretscher and Cohn proposed the two-signal model in 1970 [57]. This model proposes that lymphocytes arrive to the peripheral immune system in an immature state (i-lymphocyte), requiring two independent signals for full activation. The signal-1 is just direct antigen recognition. While the signal-2 results from the interaction of this cells with a second lymphocyte subset, referred as the helper lymphocytes. The cooperation between the helper and the immature lymphocyte was postulated to be antigen mediated, so that only cells recognizing the same antigen could cooperate to each other. If signal 1 was received alone the i-lymphocyte was permanently inactivated. Only when the two signals were properly in place an immune response could be mounted. Therefore in this model tolerance and immunity were associated respectively to the lack or the existence of helper activity (signal 2), since antigen recognition (signal 1) was always guaranteed.

To explain the self/non-self discrimination problem the model has to explain the absence of helper activity for self-antigens and its existence for non-self antigens, being precisely at this point where several hypotheses have been proposed along these years. The model was commonly complemented by clonal deletion of the helper lymphocytes, providing a simple explanation for the non-strict character of B cells deletion, given that the helper lymphocytes turn to be CD4+ T cells. But as clonal deletion became less accepted as the mechanism for natural tolerance, additional proposals were then made. An alternative idea that follows the line of the original two-signal model is due to Cohn and Langman (reviewed in [58]). These authors proposed that the Th cells were also produced in immature state (iTh). If the iTh meets an antigen that provides signal-1 in the absence of help from previously activated helper T cells they became permanently inactivated. But in the absence of antigen stimulation the iTh can be spontaneously activated in a kinetically slow process. With this simple rules self/non-self discrimination can be explained given that the self-antigen preexists the organization of the T cells repertoire, but the non-self antigen are just encountered later on in life. Note that if the antigen is always present the iTh cells are systematically inactivated consequently leading to tolerance. But if an antigen arrive later to the system then at least some of the iTh cells specific to it will have already become activated, being possible to mount the immune response.

Experimentally this theory is strongly supported in respect to the requirement for antigen mediated lymphocyte cooperation during the immune reaction (the requirement for T cell help), but lacks direct experimental support on many other postulates. The model successfully explains B-T cells cooperation in immune responses to those antigens typically referred as thymic dependent antigens. This B-Th cooperation is antigen mediated, requiring a direct cell-to-cell contact once the B cells internalize the antigen and present it in the MHC molecules to the helper T cells [59, 60]. During the Th-B cells conjugation several signals are interchanged between this cells e.g. CD40-CD40 ligand interaction and cytokine production in both sides of the conjugate [61]. For cytotoxic T cells the requirements for helper activity have been also considerably supported [62, 63]. Although the molecular mechanism for this cell interaction is less understood. The Tc-Th cooperation occurs through linked recognition by the Tc and the Th cells of the same antigen presenting cells. Either by simultaneous conjugation to the APC as proposed by Mitchitson et al [64, 65] or by "licensing" of the APC as more recently proposed [66-68]. In any case the Tc-Th interaction is antigen mediated although it is not as antigen specific as the B-Th cells interaction.

The two-signal model bring into place the concept of T cells help in the self/non-self discrimination problem, stating the notion that tolerance in most instances takes place at the level of the T helper

activity, being it sufficient to explain the lack of B or Tc reactivity. Nevertheless, following the extension by Cohn and Langman, this model is an elegant but insufficient solution for the self/non-self discrimination problem. Two major arguments can be provided in this sense. 1) There are some experimental systems where the preexistence of the antigen does not lead to tolerance, as straightforwardly predicted by the model [69-73]. For instance take the system recently described by Anderson et al [69]. In this system, RAG- mice that bear a six-month-old skin graft is reconstituted with T cells precursors from a syngenic RAG+ mice. This chimeric animal reconstitutes its immune system in the presence of the mismatch antigens in the graft, but nevertheless rejects it. 2) The second argument is a theoretical one. Given that the cooperation between T cells is not strictly antigen specific, some self-reactive iTh cells would get help from the non-self reactive helper T cells during the immune response, being consequently activated and leading to autoimmunity. This logical consequence of the theory may be reasonable since it provides an explanation for the etiology of autoimmune disorders, additional to the possible cross-reactivity of T cell receptors extensively discussed by Cohn and Langman [58]. But, in our opinion, the frequency of these events is much too high, leading to an unrealistic expectation for the incidence of autoimmune disorders. Note in this respect, that T cells cooperation is mediated by linked recognition of the APC and the non-self antigens will be always co-presented with some self-antigens in the same APC.

1.1.3.3 The innate immunity based theories for self/non-self discrimination.

This section introduces two recent theoretical proposals that base the self/non-self discrimination problem on the activity of the innate immune system. These theoretical proposals subordinate the activity of the adaptive system to the innate system. These theories are further elaborations of the original Lafferty and Cunninghan ideas [74], which are frequently merged or confused with the two-signal model in most Immunology textbooks. These authors extended the previously described two-signal model, providing an alternative explanation for the activation of the helper T cells (Th). They proposed two independent signals for the activation of Th cells. Signal-1 was antigen recognition, and signal-2 was an antigen independent signal, referred as costimulation and provided by an appropriate antigen-presenting cells (APCs). As in the two-signal model, signal 1 alone was postulated to induce cell inactivation. Under this theoretical framework, the self/non-self discrimination problem relates therefore to the availability of the costimulatory signal for the activation of the helper T cells. Self-antigens are presented by immature or non-specialized APCs that do not provide costimulation, leading to cell inactivation, while non-self antigens are presented by specialized APCs that provided costimulation leading to activation. The decision on which

antigen to react to depends on the activity of the innate immune system, that via the appropriate antigen presentation instructs the adaptive immune system response. There are two main theories of this kind. First, there is the theory proposed by Janeway et al [28, 75], where the direct recognition of microorganism-associated antigens by the innate immune receptors in the APCs lead to the APC activation, generating the proper inflammatory environment where the helper T cells can become activated. The absence of these inflammatory environment leads to tolerance or to ignorance of the given antigen. Second, there is the danger theory proposed by Matzinger et al [27, 76] where the APCs are unable to distinguish self and non-self antigens, instead they are instructed by a non-defined danger signal that is produced by normal tissue cells once damaged by pathogenic activity. In this sense the authors claimed to overrule the self/non-self discrimination problem, substituting it by the problem of distinguishing innocuous from dangerous antigens.

Considerable experimental support exists for the role of costimulation in the activation of T cells in accordance with the ideas of Lafferty and Cuninghan. These costimulatory signals have been molecularly characterized. For instance the B7 molecules superfamily [77] has been extensively studied on its costimulatory activity through the signaling via the CD28 molecule in T lymphocytes [78]. The requirements of coestimulation in the activation of T cells have been particularly well demonstrated for the IL-2 gene expression *in vitro*. Schwartz and coworkers [79] showed that T cell stimulation in the absence of costimulatory signal, blocks their production of IL-2 and thus blocks cell proliferation. Moreover these cells remain unresponsive to further stimulation, even in the presence of costimultory signals. This unresponsive state is referred as anergy and has been conceptually generalized as a state of unresponsiveness induced in T lymphocytes after interaction with APC in the absence of appropriate costimulation. Nevertheless this state has been difficult to prove *in vivo* and it appear most of the time as a transient effect that can be overcome by the addition of IL-2 *in vitro* [80]. Experimental data has been also accumulated pointing to the activated dendritic cells (DCs) as the professional APCs referred by Lafferty et al. (reviewed on [81]). Dendritic cells are derived from blood leucocytes and expressed in most normal tissue in an immature state, which is characterized by a high phagocytic capacity and a low expression of MHC-II and costimulatory molecules. These immature dendritic cells can be activated *in vitro* by cytokines, stress proteins and bacteria derived products such as LPS and others. Once activated they migrate to the proximal lymph node expressing high levels of MHC and known costimulatory molecules and lose their phagocytic capacity. Activated but not immature dendritic cells are potent activators of T cells *in vitro* through specific antigen presentation. Nevertheless grasping the role

and importance of these dendritic cells in self/non-self discrimination will require better studies of their physiology.

As a positive aspect, these theories have recalled the interest of immunologists in the activity of the innate immune system, leading to a deeper study of the receptor and mechanisms in this system. Moreover these theories provide a simple mechanistic interpretation for the role of adjuvants, which are immunopotentiating substances so far empirically used in Immunology. In this view adjuvants directly activate the innate immune system, promoting an inflammatory environment that favors the immune reaction. Nevertheless, despite the logical consistency of these theories, it is hard to believe that the innate immune system could hold the solution for the self/non-self discrimination problem. Two major arguments can be made in this sense.

The first argument is a theoretical one. These theories propose a unidirectional dialog between the innate and the adaptive immune system, the innate mechanisms instruct the adaptive mechanisms on when and how to respond to a given stimulus. In biology, however, most of the interactions between organism components appears rather like a bi-directional dialog where each component is intimately related to the activity of the other. Consider the well-known example of the interaction between the nervous and the endocrine system. In this case the nervous system can control endocrine activity via the hypophysis gland, just smelling food leads to the secretion of bilear glands. But the activity of the endocrine system also affects nervous system activity. For instance a castrated dog, lacking the secretion of sexual glandules, become less aggressive altering in this way its psychological behavior. Following this rule it must be expected the same type of bi-directional dialog between the innate and the adaptive immune system. These theories emphasized to the extreme how the innate activity affects the adaptive immune activity, but little is regarded in the interactions on the other direction. Nevertheless there are many remarkable examples of such interaction. First of all there is the well-known role of antibodies in directing complement activity, or the role of IgE antibodies in mastocytes activation in allergic phenomena [2]. But more interestingly there is growing evidence for the modulation of the dendritic cells maturation and functions by T cell activity. On the one hand, the cognate interaction between the DC cells and the T cells, mediating a CD40-CD40L interaction, appears to up-regulate the expression of coestimulatory molecules and the production of IL-12 by the DC cells [82, 83]; and on the other hand the cytokines produced by activated T lymphocytes appears to modulate the activity of DC cells in a quite complicated fashion, depending on the particular subset of DC cells studied [81, 84]. For instance IL-10 inhibits monocytes differentiation [85] into dendritic cells; IL-4 together with

GMSF promote the maturation of myeloid dendritic cells [83] and Il-3 is a survival factor for lymphoid dendritic cells [86]. Moreover there are some experimental observations indicating an impaired maturation of the dendritic cell populations in animal models lacking mature T cells [87]. We believe that more information in this direction will be accumulated, as the physiology of the different subsets of dendritic cells is further studied.

The second argument against these theories is the existence of several experimental systems where the activation of the innate immune system is difficult to argue as either a necessary or a sufficient condition for the immune reaction. That the activation of the innate immunity is not sufficient to trigger immune responses may be argued from the difficulties to raise these responses against self-antigens even with the use of adjuvants. But this argument should be drawn carefully, since following the discovery of Freund's adjuvant several experimental animal models were established, where adjuvant alone or adjuvant + self-proteins revealed autoreactivity, inducing autoimmune diseases (reviewed in [88]). Nevertheless we must note that those models are restricted to a few self-antigens and some particular genetic background of the animal. Moreover diseases induced in these models are always transient. Once recovered from the autoimmune episode the animals become resistant to further disease inductions [88], although these secondary inductions also use adjuvants. The mechanism mediating the resistance induced by this treatment is still largely unknown, but in some particular models like in the experimentally induced encephalomyelitis (EAE) it has been established an active role for T lymphocytes in mediating this phenomenon [89-92]. That the innate immunity activation is not always necessary for a successful immune reaction can be argued for instance in the studies of the non-obese diabetic mice (NOD). Insulin dependent diabetes is developed spontaneously in NOD females raised in clean conditions, specific pathogen free conditions (SPF) for the specialists [93, 94]. But strikingly they do not develop diabetes if they are raised in non-clean condition (not SPF). So letting them exposed to normal pathogens that continuously activate their innate and adaptive immune system lead to their protection from autoimmunity. Another interesting example is the experimental model recently developed and reported in the Matzinger group [69]. They construct a chimeric animal by reconstituting RAG- females H-Y mice with bone marrow T cells precursors from the RAG+ congenic counterpart. This animal reconstitutes its immune system in the presence of a six-month old skin graft from a male or female H-Y syngenic animal. Interestingly, although the graft is perfectly integrated to the animal skin, the chimera systematically rejects the male graft but tolerate the female one. The authors of this report try to accommodate the latter results into the danger theory, searching for any differences between graft and normal skin. But they only report a difference in mRNA expression of

constitutive protein GAPDH, which may reflect a lack of technical standardization rather than an immunologically relevant difference. In the systems exemplified above, as in many others, it is difficult to claim a determinant role for direct innate immune recognition or for tissue damage (danger) in initiating the immune reaction.

1.1.3.4 Theories based on active (dominant) tolerance.

The theories described in the previous sections have as a common hallmark that either deletion or inactivation of specific lymphocytes (B, Tc and Th) explains tolerance, and the interaction between lymphocytes, if relevant, is always a positive cooperation that potentiate the immune response (e.g. the classical T cell help in the two signal model). Consequently the view of tolerance in these theories has been referred as passive or recessive, since it relies in the absence of specific lymphocyte activation. In contrast to the latter, is the view of tolerance as resulting from the activation of lymphocytes that through negative and positive cooperation leads to an apparent unresponsiveness (tolerance) in the overall immune system dynamic. This alternative view of tolerance has been then referred as active or dominant [95]. Being exponents of this line of thinking the idiotypic network hypothesis proposed by Jerne et al [96, 97]; The concept of immune deviation to the Th1 or Th2 cytokines pattern in the T helper activity [98]; and the concept of suppressor T cells whose function is to inhibit the immune activity of other T cells [99, 100].

Dominant tolerance represents a major shift in immunological thinking. Note that recessive theories of tolerance go in the line of the extreme reductionism approach that has lately dominated biological sciences [101]. In this sense immunity and tolerance are reduced to the direct reactivity or unresponsiveness of the elementary components of the system, the lymphocytes. On the contrary, theories based on dominant tolerance propose a holistic view of the immune system, where the emergent properties of the dynamical interactions between lymphocytes determine the outcome of immunity or tolerance. This different theoretical framework clearly has an imprint in the understanding of the self/non-self discrimination problem. Particularly striking is the shift in the view of natural tolerance that goes from a state of complete inactivity of the system to a fully dynamic state that includes continuous lymphocyte activation and regulation. This thesis elaborates on a theory based on dominant mechanisms of tolerance, paying special attention to its implication for the self/non-self discrimination problem. Particularly we focus in a theoretical construct that considers the existence of suppressor/regulatory T cells in the immune system. A description of this theory, including the experimental phenomenology that supports it, is provided in the next section.

1.1.4 Regulatory T cells.

1.1.4.1 The founder observations on suppressor/regulatory T cell.

Gershon and Condo first reported in 1970 the existence of T cells whose function was to inhibit the activity of other T cells [99]. This seminal report opened what was called the suppression era in Immunology [102]. During this period, it was accepted that suppression was due to CD8+ T cells that exerted their function by producing antigen-specific soluble factors that bore determinates encoded by a specific region in the MHC genes and/or determinants encoded by immunoglobulin genes [100]. The failure to molecularly isolate and sequence such determinants leads to the collapse of the suppression concept in the early 80's. The "suppressor" word was deleted from immunological dictionary and the phenomenology associated to it was regarded as mystical or derived from dirty experiments [103]. Some of this phenomenology was reinterpreted following the proposal of Moller et al. [104] where CD8+ suppressors were simply viewed as cytotoxic T cells that promote the lyses of CD4+ helper T cells specific of the suppressed response.

Parallel to the studies in the suppressor era, a set of observations was accumulated, leading to the rebirth of the suppressor or, as modernly referred, regulatory T cells in the middle 90's. These were observations related to the role of thymus in the prevention of autoimmune disorders. The first of these reports dates from 1969 where Nishisuka & Sakakura [105] observed that female mice neonatally thymectomized became infertile secondary to the development of oophoritis. Thymectomy in day 3 before birth, but not on day 1 or >7, induced the disease. Thymus grafting or injection of 7-day-old thymocytes or adult spleen T cells on day 7, but not on day 40 prevented disease development. The prosecution of this study in the next 10 years established a dependence of the effect on genetic background concluding that different strains of mice develop different organ specific autoimmune diseases [106, 107]. For instance Balb/c mice preferentially develop gastritis, while C3H mice preferentially develop thyroiditis. These experiments were interpreted as the production in the thymus of T cells with the capacity to prevent the development of these autoimmune disorders. The regulatory T cells involved in this phenomenon appear later to be CD4+ T cells, and not CD8+ T cells [108, 109]. A second line of experiments pointing also to the production of regulatory cells by the thymus is due to Penhale et al 1973 [110]. This author showed that autoimmune thyroiditis follows thymectomy and partial depletion of lymphoid cells by sub-lethal irradiation in adult rats. This autoimmune disorder was prevented by transference of lymphoid cells from normal adult rats and could not be induced in euthymic animals. The cells able to prevent the development of disease turned out to be a subset among CD4+ T cells [111].

1.1.4.2 The resurrection of suppressor T cells.

A first line of experiments leading to the resurrection of the regulatory T cells in the past decade is due to the study of the post thymectomy syndrome by Sakagushi and coworkers. This author was the first in 1982 to fractionate the regulatory T cells involved in this syndrome, according to the CD5 (lyt-1) surface marker [108, 109]. This report showed that transference of CD5+ but not CD5- spleen cells from adult normal mice prevented the development of autoimmune disorder after neonatal thymectomy. Moreover transference of CD5- cells form a normal adult animal to a nu-/nu- mice lead to development of autoimmunity like those in the post thymectomy syndrome and cotransference of CD5+ population prevented disease development. This experiment clearly demonstrated the existence of autoreactive lymphocytes in a normal adult mouse that could cause autoimmune disorders, and the existence of regulatory population in the same animal capable to inhibit or suppress the pathogenic activity of these cells. A major limitation in this initial observation was that the CD5 marker is expressed in almost 80 to 90% of the total lymphocytes, considerably limiting its operational value for the study the regulatory cells. A major advance in the field followed the report by Sakaguchi et al. 1995 that regulatory activity in this system segregated with a minor 5-10% subpopulation of CD4+ T cells in the spleen. The high level of expression of CD25 characterized the regulatory fraction. This marker is the alpha chain of the high affinity receptor for IL-2 and an activation marker for T lymphocytes. Although this marker seems to be a mere correlate of the regulatory activity [100], it gives a narrow enough separation as to promote further molecular characterization of the regulatory T cells. A second step forward in this experimental system was the establishment of an *in vitro* assay that shows the suppressor activity of the CD25+ CD4+ T cells over the CD25-CD4+ T cells [112] in a good correlation to the results of the *in vivo* system. This *in vitro* assay was quite reproducible and contributes considerably to the further molecular characterization of this regulatory T cells (see next sections).

A second line of experiments that also catalyzed the resurrection of regulatory cells is the studies by Mason's group. In 1990 these researchers reported the existence of regulatory CD4+ T cells in the spleen of normal rats [111]. This regulatory T cells were characterized by the low expression of the RB isophorm of the CD45 marker, which was previously regarded as an activation/memory marker. When transferred to a nu-/nu- rat, CD4+ CD45RChigh but not CD4+ CD45RClow or total CD4+ T cells induces an autoimmune disorder similar to graft versus host disease (GVHD). The regulatory CD4+ CD45RClow fraction of the total cells were then shown to prevent [111] development of autoimmunity induced by thymectomy and sublethal irradiation in a protocol inspired in the original Penhale et al [110] observations. This experimental system was further extended to a mouse model

where inflammatory bowel disease (IBD) was induced in SCID mice by transference of CD4+ CD45RBhigh cell from normal mice [113]. This wasting disease was prevented by co-transference of the CD4+ CD45RBlow T cell population. In the mouse model, disease was shown to result from an uncontrolled inflammation to intestinal flora. In this sense several experiments showed that disease only appear when transferring CD4+ CD45RBhigh cells to mouse with intestinal flora containing Helicobacter Hepaticus [114] and not by transferring this cells into a germ free animal with no intestinal flora. Nevertheless, CD4+ CD45RBlow cells from a germ free animal where as capable to prevent the disease as those ones from a normal animal. The latter settle down the idea of a role for "self-reactive" regulatory T cells in the control of immune responses to chronic inflammations. The similarities between these models and Sakagushi model becomes more striking, given the recent demonstrations that the regulatory activity in the mouse model is further restricted to CD25+ fraction in the CD4+ T cells [115, 116]. Suggesting common base for the regulatory mechanism in these two experimental systems.

A third experimental system that also contributes to the modern view of regulatory T cells is due to the group of Waldmann. This group explored an experimental model where tolerance to minor histocompatibility antigens in a graft is induced by administration of non-depleting anti-CD4 and anti-CD8 antibodies [117, 118]. Although the same tolerogenic effects, have been subsequently demonstrated in other antibody combinations [119, 120]. Interestingly the antibody-induced tolerance in this system is permanent, antigen specific and mediated by regulatory T cells [121]. The latter was uncovered by grafting a mismatch tissue into nu-/nu- mice that have been reconstituted with cells from animal rendered tolerant or not by the antibody treatment. Animals receiving cells form non-tolerant animals; rapidly rejected the graft, while those ones receiving cells form tolerant mice accepted. Moreover mice receiving a cotransfer of cells form tolerant and non-tolerant animals also accepted the graft. The regulatory cells involved in this model where first report as CD4+ T cells [121] and in more recent studies [122] indicate that this regulatory activity is considerably enriched in CD25+ fraction of the CD4+ T cells.

The last but not the least of this founder experimental system is due to the collaborative work of the groups of Nicole Le Douran and Antonio Coutinho (reviewed in [95]). These authors studied the effect of a neonatal implants of thymic epithelium in different animal models. They showed first in the avian model and later in the mouse model that the grafting of allogenic or even xenogenyc thymic epithelium confers tolerance to graft of other peripheral tissues such as skin, heart and kidney. Again, this form of tolerance, was mediated by regulatory CD4+ T cells [123], since the

transference of these cells into an empty animal also transfers the tolerant status. This experimental system clearly suggested a role for thymus and particularly for thymic epithelium in the generation and selection of regulatory T cells in opposition to the classical role of thymus in clonal deletion. In contrast with the previous systems, it is still unclear whether the regulatory T cells in this experimental system are enriched within the CD25+ subset.

Following these pioneer experimental systems, regulatory T cells became again popular in Immunology, being this evident from the explosion of papers and reviews that appear in the last two years in the field (reviewed in [124-128]). Most emphasis has been put on the CD4+ CD25+ regulatory T cells although many other regulatory phenotypes have been lately described. For instance: CD4+NKT Cell [129], CD4-CD8- T cells [130], CD8+ T cells [131]. Recent studies on CD4+CD25+ T cells have considerably expand the phenomenology associated to these cells, showing for instance that they migh be present in humans [132-135], that they might be important in the control anti-tumor response [136, 137], that they might control T cells homeostasis [138]), that they might be relevant for the tolerance to transplantation antigens [139, 140] and that they might be induced or selectively expanded by oral immunization [141]. Many aspect of the normal physiology and mechanism of action for these cells have been also uncovered. Those aspects more relevant to understand this Thesis are briefly reviewed in the next sections.

1.1.4.3 The generation of CD4+CD25+ Regulatory T cells.

Regulatory CD4+CD25+ T cells are generated in the thymus, being then subsequently exported to the peripheral immune system. Some of the founder experiment in the suppressor T cells field already pointed in this direction, by showing dependence of the prevention of autoimmune disorders with the presence or absence of thymus (reviewed in [142]). But more recent experiments have clearly reinforced this idea. In this sense CD4+CD25+ thymocytes show efficient regulatory properties both *in vivo* and *in vitro* [143]. And two recent papers by Jordan and coworkers [55, 144] molecularly characterized the thymic generation of CD4+CD25+ regulatory T cells. In these reports, a clonotype+ CD4+ CD25+ with regulatory activity were found in the thymus and the periphery of double-transgenic mice expressing a transgenic TCR that recognize the S1 peptide of influenza haemagglutinin. Generation of this regulatory population requires specific antigen recognition in radio-resistant cells of the thymus (mainly thymic epithelium) with a high affinity of TCR-MHC peptide interaction, since in a second transgenic with a TCR of lower affinity for S1, the regulatory T cells were not observed. These results demonstrated the requirement of specific antigen recognition in the thymic generation of regulatory T cells, suggesting that this process

requires high affinity MHC-TCR interaction in the thymus, in agreement with a theoretical model proposed by Modigliani et al [145]. Although more experiments, perhaps involving several transgenic animals, are required to demonstrate whether this finding indeed correlates with the lower TCR-MHC peptide affinity in the transgenic mice and it is not just the consequence of any other unsuspected difference between the transgenic animals.

An important and as-yet unresolved question is whether CD4+CD25+ regulatory T cells are just a distinct T cell lineage whose function is determined in the thymus or whether they can also be derived from the differentiation of naïve CD4+ T cells at the periphery. In the experimental model of Waldmann and colleagues, where tolerance to peripheral antigens is induced by monoclonal antibodies treatment, the coexistence of regulatory and naive CD4+ T cells in nu-/nu- recipient for two weeks in the presence of antigen, resulted on the transference of the regulatory capacity to the previously naïve T cells population [118]. This observation recalled the term infectious tolerance and it was interpreted as a peripheral differentiation of the naïve CD4+T cells to the regulatory phenotype. Moreover in the experimental system of Coutinho and colleagues [146], infectious tolerance was also documented but this time only recent thymic emigrants CD4+ T cell populations where capable to acquire regulatory properties. Nevertheless, none of the observations above proves the extrathymic generation of regulatory T cells, since they can be always interpreted by the expansion of a small preexistent regulatory T cells population.

1.1.4.4 Specificity and peripheral maintenance of regulatory CD4+CD25+ T cells.

Little advance has been achieved in determining the fine antigenic specificity of regulatory T cells, perhaps due to the difficulties in isolating and cloning these cells. On the one hand a repertoire analysis of the CD4+ CD25+ T cells has shown that they represent a cell population as diverse as the CD4+ CD25- T cells at least in term of variable region genes usage [112] and CDR3 length distribution [147]. On the other hand, indirect experimental data suggests that regulatory T cells recognize self-antigens in the peripheral tissues, being this recognition required for the maintenance of regulatory cells activity in the periphery. A pioneer experiment from Taguchi & Nishizuka [148] in 1981 showed that spleen cells from normal adult male mice were more efficient suppressors of autoimmune orchitis in the post thymectomy syndrome (post-3dTx) than were spleen cells from female mice or male mice that had undergone neonatal castration. This lack of suppressor activity was specific, since spleen cells from male or female mice or neonatally castrated male mice where comparable efficient to prevent autoimmune gastritis in the post-3dTx syndrome. Studies in prostatitis then showed that the differences were more relative than absolute, since adding 10 times

more spleen cells from female mice was as efficient as the male spleen cells in preventing the autoimmune prostatitis. In the same line of results, a recent experiment by Mason et al 1999 [149] showed, that thymocytes but not peripheral CD4+CD45RClow cells from rats render neonatally athyroid were capable to prevent thyroiditis induced in adult rats following thymectomy and sublethal irradiation. The loss of peripheral regulatory T cells was specific for the extirpated organ as T cells from the athyroid animals could prevent the development of diabetes.

In summary, as a whole the results reviewed in this section indicate that regulatory CD4+CD25+ T cells are produced in the thymus and their persistence and/or expansion in the periphery requires specific recognition of self-antigens. Nevertheless there are still more questions than answers in this issue. For instance, how diverse is the self-antigen repertoire recognized by the regulatory T cells? Or whether the antigens that mediate the selection of regulatory phenotype in the thymus are the same recognized lately in the periphery? Recently Sakagushi and coworkers has reported the first successful attempt to clone regulatory T cells [124]. It must be expected that this achievement could help to enlight many aspects on the specificity and homeostatic requirement of regulatory T cells.

1.1.4.5 Mechanisms of action of the Regulatory T cells.

Several aspects of the mechanism by which the regulatory CD4+ T cells in general, and CD25+ T cells in particular suppress their targets have been elucidated by recent *in vivo* and *in vitro* studies. Waldmann et al reported [121, 150, 151] that mice rendered tolerant to skin graft from mouse strain A, by regulatory T cells induction with monoclonal antibody treatments, rejected skin grafts from a strain B but tolerated skin grafts from F1 mice that bear antigens from both A and B strains. Moreover after tolerating F1 skin the animals became also tolerant to the skin graft from the strain B, suggesting that coexpression of antigen determinants from A and B in the F1 skin induced tolerance to the previously rejectable antigens in the B skin. Interestingly tolerance induction require that both A and B antigens were expressed in the same cells, since simultaneous adjacent skin graft form strain A and B were respectively tolerated and rejected in this model. Overall, these experiments bring to the field the concept of linked suppression or linked regulation. In this view regulatory T cells specific for antigen A indistinctly suppress the immune reaction to all the antigens coexpressed with antigen A in a given tissue. This type of "linked suppression" was investigated by several *in vitro* systems, from systems using T cell lines [152, 153] to *ex vivo* systems where suppression *in vivo* and *in vitro* is well correlated [112, 154]. These studies, with the exception of two recent controversial results [155, 156], indicated that suppression mediated by regulatory T cells *in vitro* requires direct cell to cell contact between antigen presenting cells

(APCs), regulatory T cells and T cells that are targets of regulation [112, 154, 157]. These observations led to the hypothesis that suppression takes place while the regulatory T cell and its target T cell are co-conjugated with a single APC, i.e. suppression requires interactions between three cell types in multicellular conjugates. Alternatively, the regulatory cells may suppress the capacity of APCs to activate the effector cells [158-161], avoiding the requirement for simultaneous conjugation. The attempts to discriminate experimentally these different hypotheses hitherto failed to provide a definitive picture. Nevertheless, the fact that *in vitro* suppression assays are typically performed using "fixed" or irradiated APC renders the suppression via the APC rather implausible.

Despite the strong constraint that the linked recognition hypothesis imposes on the mechanism by which regulatory T cells suppress their targets, it is not yet clear what are the molecular mediators of this interaction or the detailed nature of this mechanism. Suppression may be the result of simple competition for activation resources provided by APC [152, 162]. Regulatory and effector cells may exchange signals while they are simultaneous conjugated with the same APC. Thus it has been suggested that regulatory cells may give an inhibitory signal to the responder T cells [143, 163], or alternatively the responder cells may become themselves regulatory cells during this interaction [118, 146].

Controversial results exist also on the role of cytokines in T cells mediated regulation phenomena. In different experimental models cytokines such as IL-4, IL-10 and TGF-β has been report to mediate the suppressive activity *in vivo* [164-166]. The experimental model for IBD induced by transference of CD45RBhigh cells into SCID mice is remarkable in this sense. In this model autoimmunity control by CD45RBlow T cells is prevented by administration of anti IL-10 or anti TGF-B antibodies [164, 165]. In opposition to these results neither anti-IL-4, anti-IL-10 or anti-TGF-β antibody administration prevent regulation mediated by CD4+CD25+ T cells in the post-3Tx syndrome [124]. Moreover CD4+CD25+ T cells from the IL-10 knockout mice were as efficient regulators as those from normal mice in preventing autoimmunity in this model. Additionally *in vitro* suppression has been reproducibly demonstrated independent of most known cytokine activity [112, 154, 157]. The latter discrepancies derives perhaps from a role of cytokines such as Il-10 or TGF-β in the activity of other cells types in the *in vivo* system interfering with particular aspect of the given disease model. Particularly IL-10 and TGF- β are known as anti-inflammatory cytokines that act for instance on monocytes and dendritic cells. Other possibility is that these cytokines have a role on proliferation, maturation or survival of the regulatory T cells in *in vivo* rather that in the suppressive interaction per se [124]. Finally, an alternative solution to this

puzzle, at least for the particular case of TGF-β, has been proposed by Nakamura et al [167], based on the existence of a membrane form of this cytokine that may indeed mediate the suppressive interaction, by a mechanism requiring cells to cell contact

Recent studies have also uncovered a role for CTLA-4 antigen in the suppressive interaction. CD4+CD25+ regulatory T cells over-express the CTLA-4 antigen on their surface [115, 168]. This may be important functionally, as anti-CTLA4 antibody abrogate the capacity of these cells to inhibit IBD [115] upon transfer of CD25- cells into SCID mice and to inhibit gastritis induced in the post-3Tx syndrome [168]. *In vitro* studies also show that anti-CTLA4 antibodies or Fab fragments abrogate the suppressive capacity of CD25+ T cells [115, 168]. Although some controversy exist in these results given that CD25+ T cells from a CTLA-4 knockout mice exhibited a suppressive capacity similar to that of CD25+ cells from a normal mice. Currently it is not known how CTLA-4 is involved in the function of regulatory T cells. But some authors [124] favor the idea that it participates in the process of activation of the regulatory T cells by the APC, which is required for the suppressive activity.

1.1.5 Regulatory T cells and the self/non-self discrimination problem.

The phenomenology, documenting the existence of regulatory T cells, represents an anomaly in all the theories for self/non-self discrimination described above (sections 1.1.3). Particularly, Sakaguchi's experimental system demonstrates the existence of T cells in the peripheral immune system of normal adult mice, which provoke autoimmunity upon transfer to an empty recipient in the absence of any known particular bacterial infection or cellular stress (danger associated signals) to mediate their activation. Moreover it demonstrates that this pathogenic activity is prevented by the action of other lymphocytes, the so-called regulatory T cells. Altogether these experimental facts cannot be accommodated in a theory that conceives tolerance as a passive, recessive phenomenon. Instead they call for a theoretical view of tolerance and particularly natural tolerance based in a quantitative balance among two T cells population, those potentially pathogenic (effector T cells) and the regulatory T cells.

The theoretical constructs emerging from these experimental systems assume that the CD4+CD25+ regulatory T cells are central players in natural tolerance, perhaps complementing other mechanisms of dominant tolerance (e.g. other phenotypes of regulatory T cells) and other mechanisms of recessive tolerance (e.g. the classical central deletion). These regulatory T cells are at least primarily generated in the thymus and they act over normal helper T cells (CD4+CD25-),

through linked recognition preventing either their activation, or their expansion in the course of the immune reaction. Whether these dynamic interactions lead to tolerance or to immunity is just a quantitative matter of how many effectors T cells are pulling toward immunity and how many regulatory T cells are pulling toward tolerance. So in these theoretical frameworks self/non-self discrimination necessarily implies a low activity of regulatory T cells in response to non-self antigen and a high activity in responses to self-antigens.

The simplest proposal to explain self/non-self discrimination would be then, that there are no regulatory T cells specific for the non-self antigens, but even under this extreme assumption it is not obvious that this theory could fulfill this task. Note that if the regulatory T cells act through linked recognition and the non-self antigen are always co-presented with self-antigens in the same APCs during the immune reaction, then those regulatory T cells specific to self-antigen might also prevent the response to the non-self antigens. Interestingly some of the experimental systems in regulatory T cells fields have clearly documented the activity of these cells in the immune response to chronic bacterial infections [113, 169-171]. So additional elaboration is required in this theory to explain self/non-self discrimination and to finally derive all the consequence of this dominant mechanism of tolerance in the immune system. To address these issue, in this Thesis we develop and study some mathematical models for the interaction between regulatory and effector T cells. Following such bio-mathematical approach was motivated by the intrinsic quantitative nature of this biological problem. The next section provides a brief, but necessary, introduction to the mathematical modeling techniques.

1.2 Mathematical modeling.

1.2.1 What's a mathematical model?

A mathematical model may be defined as a representation or description of an experimentally delineated phenomenon, by means of mathematics, with a view to capture the most salient (essential) aspects of the phenomenon at hand.

But the above definition is somewhat vague, since not every mathematical description of an experimental phenomenon would be commonly referred as a model. There are two extremes in the mathematical description of a phenomenon. On the one hand, we could simply list what we observed in experiments. These often involve mathematical structures, notably measurements (number +units), and we could primarily regard this numbers as a mathematical representation of

the experiment. On the other hand, modern physics resolves around a small set of 'first principles' from which, through the application of a considerable mathematical apparatus a wide range of physical phenomena are described, most of the time with considerable accuracy. The term "models" is typically reserved for the situation between these two extremes. The model is more ambitious than a simple quantitative description of experiments, but it is not as deep and general as the first principle description. The model is somehow the halfway house where first principles are not available, or their application is not feasible, yet some general ideas about the phenomenon study are properly incorporated in the model.

1.2.2 What can mathematical models do for you?

Models are built to address particular questions about the object, system or phenomenon studied, so they are designed for a given purpose. Models are just a simplified caricature of the real world and they are only a good description of the study phenomena in a limited context. Models are built based on some knowledge about the studied system. This knowledge is resumed in the form of postulates, which are then translated into the mathematical formalism of the model. Postulates can be a well-established aspect of the real world or just some hypothesis under test. But once properly formulated, the model can be used to derive the ultimate consequences of its postulates.

Mathematical models do nothing else but establishing logical statements of the form if this proposition then that consequence. Particularly, they establish what is entailed by their postulates. Consequently for each postulate of the models, we could then ask how relevant it is for the overall model behavior? And how well founded is it on experimental data? Following this procedure, those bits of the model, which are essential for a given aspect of the model behavior, are identified, providing an important insight on the studied phenomena. Additionally since the mathematical formalism of the model can derivate all the consequence of a given set of postulates, they sometimes leads to the prediction of unexpected properties of the studied system, suggesting new experimental designs or problems to be explored. Another possibility is to use the models to perform logical falsification and hypothesis testing. Note in this sense, that mathematical model cannot be proven true from comparing to experimental observations, but they do can be proven false. If every model prediction is consistent with experimental data on the real system, then all we can say is that the model has not been falsified or is compatible with the data. But if some experimental data is inconsistent with the model then we can reject the model. So by studying several alternative hypothetical models and comparing them to experimental data we could eliminate those ones, which are inconsistent, narrowing down the number of alternative models.

Nevertheless this latter use of mathematical models should be applied carefully, since no mathematical model is absolutely true and if provided sufficient detailed data from the real system they would most probably be proven false.

1.2.3 Mathematical models in Immunology and in this Thesis.

Mathematical models are often used in biological sciences. There are some branches of biology such as ecology, epidemiology and neurobiology where mathematical modeling are nowadays part of the organic life of the field. In Immunology the use of mathematical modeling started with the reports of George Bell in 1970 [172, 173], where a simple model for clonal selection theory and antibody production was formulated and studied. Since these pioneer reports many mathematical models have been developed on the field, although the impact of modeling has remained very limited. Perhaps due to the fact that modelers and experimentalist are still quite separate communities, having communication difficulties. Some of the immunological problems, which have been frequently addressed by modelers are: the mechanism mediating immunological memory [174-178]; the mechanism that control homeostasis of lymphocyte populations [179-186]; the processes of lymphocyte selection and the diversity of the immune repertoire [180, 187-193]; the mechanism of lymphocyte activation and proliferation [194-202]; the dynamic of viral infections, such as HIV [203-206] and others [182, 207-212]; and the central issue of tolerance and self/non-self discrimination in the immune system [213-223]. The theoretical thinking expressed by the models has followed the evolution in immunological theories. Actually most of the theoretical ideas, hypotheses, which have dominated immunological thinking in the last years, have been explored by mathematical modeling, aiming to derive their most remote and quantitative implications. For instance in the case of the self/non-self discrimination problem, there are several models: exploring the implications of clonal selection theory from a quantitative analysis of the diversity of peripheral lymphocyte repertoire [187, 188, 191, 224]; studying the dynamic of class differentiation within the Th1- Th2 paradigm [220, 221, 225]; exploring the idiotypic network theory [215, 217, 218, 222, 223, 226]; and there are also some models including the figure of suppressor or regulatory T cells [219, 227-231].

The models in the present Thesis are inspired in many of these previous works, adopting some of the usual assumptions and formalisms developed there. Nevertheless the novelty of the concept of linked suppression and the hypothesis of the interaction between regulatory and effector T cells requiring the formation of multicellular conjugates, posed us the problem of formulating a new kind of mathematical model for this interaction. This type of interaction was not available in pre-existing

models, which typically consider either a direct cognate interaction between cells [219, 232-235] or cell interactions mediated by the secretion of some soluble factor, i.e. some cytokine [220, 221, 225]. Technically the models in this Thesis can be classified as population dynamics models. They describe the time evolution of the sizes of several lymphocyte populations (i.e. the population of effector and regulator cells or different lymphocytes according to their antigenic specificity), which are represented as variables in a set of ordinary differential equations (ODEs). This system of ODEs makes some explicit assumption about the interaction between the cells, exploring their consequence to the overall dynamic of the cell populations.

1.3 About This thesis.

1.3.1 The aim, the approach and the objectives

The aim of this thesis is to further our understanding on the implications of dominant mechanism of tolerance for the immune system. Specifically we focus on the study of T cells mediated suppression as one of these mechanisms.

Our approach is a bio-mathematical approach. We develop and study mathematical models and confront these models with experimental data. We proceed in two complementary directions. On the one hand, we try to further our understanding of the detailed mechanism by which regulatory T cells mediate their function. Here the models are used to effectively compare alternative candidate mechanisms existent in the literature. The pivotal working hypothesis of this work is that the interaction between the Regulatory T cells (CD4+ CD25+) and their target cells (CD4+ CD25-) depends in the simultaneous conjugation of these cells with the same antigen-presenting cell (APC). On the other hand we look for the implications of T cell-mediated suppression for the organization and operation of the immune system. Particularly we explore whether this model can provide a successful explanation for the self/non-self discrimination problem and its implication to understand the etiology of autoimmune diseases. Here the models are used as tools to bridge levels of organization, to understand the implication at the systemic level of some postulated interaction between individual cells.

To fulfill our goals we designed the following set of practical objectives:

1. To develop a mathematical model describing the dynamic of two populations of cells whose interaction depend on their simultaneous conjugation to a third host cell.

2. To apply the methodology developed to study the dynamics of a population of regulatory cells (R) and a population of their targets (E), that interact at the surface of antigen presenting cells (APCs) according to each one of the following candidate models:

    - There is no direct interaction between R and E on the surface of the APC. They are just competing for the limited number of antigenic sites on the APC surface.
    - The R cells deliver an inhibitory signal to the E cells.
    - The R cells deliver an inhibitory signal to the E cells, and receive a stimulatory signal from them.
    - The R cells induce the differentiation of the E cells to the regulatory phenotype.

3. To analyze the quantitative implications of these models, contrasting the results with the results of *in vitro* and *in vivo* experiments in the dominant tolerance field.

4. To build a numerical simulator of the immune system, that using as core dynamics equations those ones studies in points 2 and 3, can extend the models to include the existence of several R and E population differing on their antigenic specificity

5. To investigate by simulations under which condition the model can account for a successful self/non-self discrimination in the immune system.

6. To study the implications of the model to understand the etiology of autoimmune diseases.

1.3.2 The Outline of this thesis

This Thesis is composed of 3 chapters, General introduction, Results and General discussion. The results presented in this thesis are contained in 5 independent scientific publications, each one in a corresponding section of the chapter of results. Sections 2.1 to 2.3 investigate the mechanism of action of regulatory T cell. Particularly, section 2.1 fulfills our objective 1, 2 and the part of 3 concerning *in vivo* experiments, while sections 2.2 and 2.3 fulfill the second part of objective 3 concerning *in vitro* experiments. The remained sections 2.4 and 2.5 investigate the implication of T cells mediated suppression in the general context of the immune system. Section 2.4 fulfills our objectives 4 and 5, while section 2.5 fulfills our objective 6.

1.4 References

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Chapter 2

RESULTS

J. theor. Biol. (2000) **207**, 231–254
doi:10.1006/jtbi.2000.2169, available online at http://www.idealibrary.com on IDEAL®

Modelling T-cell-Mediated Suppression Dependent on Interactions in Multicellular Conjugates

KALET LEÓN*†‡, ROLANDO PERÉZ*, AGUSTIN LAGE* AND JORGE CARNEIRO†

**Centro de Immunología Molecular, P.O. Box* 16040, *Habana* 11600, *Cuba and*
†*Instituto Gulbenkian de Ciencia, Apartado* 14, 2781-901, *Oeiras, Portugal*

(*Received on* 7 *February* 2000, *Accepted in revised form on* 10 *August* 2000)

Tolerance to peripheral body antigens involves multiple mechanisms, namely T-cell-mediated suppression of potentially autoimmune cells. Recent *in vivo* and *in vitro* evidence indicates that regulatory T cells suppress the response of effector T cells by a mechanism that requires the simultaneous conjugation of regulatory and effector T cells with the same antigen-presenting cell (APC). Despite this strong requirement, it is not yet clear what happens while both cells are conjugated. Several hypotheses are discussed in the literature. Suppression may result from simple competition of regulatory and effector cells for activation resources on the APC; regulatory T cells may deliver an inhibitory signal to effector T cells in the same conjugate; or effector T cells may acquire the regulatory phenotype during their interaction with regulatory T cells. The present article tries to further our understanding of T-cell-mediated suppression, and to narrow-down the number of candidate mechanisms. We propose the first general formalism describing the formation of multicellular conjugates of T cells and APCs. Using this formalism we derive three particular models, representing alternative mechanisms of T-cell-mediated suppression. For each model, we make phase plane and bifurcation analysis, and identify their pros and cons in terms of the relationship with the large body of experimental observations on T-cell-mediated suppression. We argue that accounting for the quantitative details of adoptive transfers of tolerance requires models with bistable regimes in which either regulatory cells or effectors cells dominate the steady state. From this analysis, we conclude that the most plausible mechanism of T-cell-mediated suppression requires that regulatory T cells actively inhibit the growth of effector T cells, and that the maintenance of the population of regulatory T cells is dependent on the effector T cells. The regulatory T cell population may depend on a growth factor produced by effector T cells and/or on a continuous differentiation of effector cells to the regulatory phenotype.

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1. Introduction

Several mechanisms contribute to immunological tolerance to body tissues. Thymic deletion purges the emergent T cell repertoire from those lymphocytes that recognize, with high affinity or avidity, peptides expressed intrathymically (von Boehmer, 1991; Huang & Crispe, 1992). Mechanisms such as T cell ignorance (Ohashi *et al.*, 1991), T cell anergy (Schwartz, 1997) or clonal exhaustion (Moskophidis *et al.*, 1993; Rocha *et al.*, 1995) may contribute to the peripheral unresponsiveness to tissue-specific antigens, which are not expressed in the thymus. However,

‡Author to whom correspondence should be addressed. Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras Codex, Portugal. E-mail: kalet@ict.cim.sld.cu, kleon @igc.gulbenkian.pt

0022-5193/00/220231 + 24 $35.00/0

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these mechanisms seem to be insufficient to explain tolerance to continuous stimulation by self-antigens, as they can easily be overcome during the course of immune responses and inflammation (Cahill *et al.*, 1997). Dealing with this chronic stimulation by peripheral antigens seems to call into action yet another, maybe complementary, mechanism of tolerance, based in regulatory lymphocytes that actively suppress (auto) immune responses. The latter form of tolerance has been uncovered by adoptive transfers to naïve recipients of T cells of donors that are either tolerant or responsive to some antigen. If in many instances the recipient becomes responsive to the antigen, in others they are reconstituted but display the "phenotype" of the tolerant donor (Sakaguchi *et al.*, 1985, 1995; Fowell *et al.*, 1991; Smith *et al.*, 1992; Modigliani *et al.*, 1995; Powrie *et al.*, 1997). By analogy with the phenotype inheritance in genetics this form of tolerance, which can be transferred by T cells, has been named dominant in opposition to the previous mechanisms that have been named recessive (Le Douarin *et al.*, 1996).

Although many different experiments of adoptive transfer of tolerance reveal the existence in normal healthy individuals of a CD4 + T cells subpopulation with suppressive properties, these regulatory T cells have not yet been isolated or cloned. This has prevented the full characterization of their phenotype and of their mechanism of action. Nevertheless, some clues on how the regulatory CD4 + T cells suppress the response of other cells have been derived from well-correlated *in vitro* and *in vivo* experiments (Takahashi *et al.*, 1998; Thornton & Shevach, 1998). These studies suggest that T-cell-mediated suppression is not mediated by soluble factors and requires cell-to-cell contact. Moreover, these studies indicate that regulatory CD4 + T cells can only suppress the response by other cells if the ligands of both cells are expressed by the same antigen-presenting cell (APC) (Cobbold *et al.*, 1996; Davies *et al.*, 1996a; Frasca *et al.*, 1997; Wise *et al.*, 1998). Because of this fact, the mechanism of regulation was baptized "linked suppression". Despite this strong requirement, it is not yet clear what the nature of this mechanism is. Suppression may be the result of simple competition for activation resources provided by APC (Lombardi *et al.*, 1994; Waldmann & Cobbold, 1998). Regulatory and effector cells may exchange signals while they are simultaneously conjugated with the same APC. Thus, it has been suggested that regulatory cells may give an inhibitory signal to the responder T cells (Suri-Payer *et al.*, 1998; Itoh *et al.*, 1999), or alternatively the responder cells may themselves become regulatory cells during this interaction (Qin *et al.*, 1993; Modigliani *et al.*, 1996a). Alternatively, the regulatory cells may suppress the capacity of APCs to activate the effector cells (Taams *et al.*, in press; Cederbom *et al.*, 2000), avoiding the requirement for simultaneous conjugation. The attempts to discriminate experimentally these different hypotheses hitherto failed to provide a definitive picture. Nevertheless, the fact that *in vitro* suppression assays are typically performed using "fixed" APC renders the suppression via the APC rather implausible. The aim of the present article is to further our understanding on the mechanisms of linked suppression and its role in dominant tolerance, by exploring its putative requirement of a simultaneous interaction between three cell types. This sets up another goal, that is to develop a model of interactions in multicellular conjugates.

Mechanisms of intercellular cooperation involving the interaction of more than two cells simultaneously have been suggested before in Immunology (e.g. the classical view of cooperation between CD4 and CD8 T (Mitchison & O'Malley, 1987; Mitchison, 1990), but they are not common in biology. No mathematical model has been developed to study the quantitative implications of such interactions on cell population dynamics. Available mathematical models of T-cell-mediated suppression have addressed the possibility that this suppression is mediated by soluble factors, as in the class regulation models of Fishman & Perelson (1994) and others, or they have addressed the possibility of suppression mediated by a direct cell contact between a suppressor clone and an antigen-specific responder clone, as in classical idiotypic network models (Cohen & Atlan, 1989; Perelson, 1989) or in the more recent T cell vaccination models (Borghans & De Boer, 1995; Segel *et al.*, 1995). Here we report the development and analysis of a mathematical model, in which the regulatory

interactions between T cells take place only in multicellular conjugates with their APC. We use this model to study the implications of this interaction on linked suppression and dominant tolerance.

2. The Basic Model

2.1. BIOLOGICAL SYSTEM AND BASIC POSTULATES

The biological system that we are going to model has the following cellular components: the responder T lymphocytes that upon activation will trigger effector function (heretofore referred to as "effectors" and abbreviated as E), the regulatory T cells, (R), and the APCs (A). The major postulates in the model are:

(1) Regulatory T cells (R) and effector T cells (E) interact only during simultaneous conjugation with an APC.

Since we are interested in studying the quantitative implication of interactions amongst T cells in multicellular conjugates with APC, we impose that in the model. We note that T cell suppression may be mediated by other mechanisms. Anti-idiotypic regulatory cells may conjugate directly with the effector cells and suppress them; regulatory cells can synthesize suppressive cytokines acting on the effector cells in a paracrine fashion; and regulatory cells can act on the APCs inhibiting their capacity to stimulate effector cells. A comparative study of these alternative mechanisms represents a broader research plan, for which this is the first step.

(2) Antigen-presenting cells are a homogeneous population with fixed size.

The population of APCs *in vivo* is certainly not homogeneous (Cella *et al.*, 1997). First, it is formed by cells of different lineage, which exhibit different capacities to stimulate T cells. Second, APCs from the same lineage may differ on the level of expression of the relevant antigenic peptide, and may be in different states of activation and/or differentiation (Sprent, 1995; Kapsenberg *et al.*, 1999). Even *in vitro* it may be difficult to achieve homogeneity of the APCs. Also, *in vivo* the total number of APCs may change and is perhaps a function of the T cells themselves (Takashima & Kitajima, 1998). This postulate allows us to obtain a simpler mathematical model, which hopefully captures those features of the real system depending on the average properties of the APC population. Those properties that depend on the APC heterogeneity are beyond the scope of the present work and may represent an area for future investigation.

(3) Each APC has a finite and fixed number of conjugation sites, which can be occupied by a single cell, irrespective of its phenotype being either regulatory or effector.

De Boer & Perelson (1994) introduced the concept of antigenic site to study T cell–APC interactions. The notion that the number of T cells that can be simultaneously conjugated with a single APC is limited is an intuitive and reasonable one: there must be some sort of steric hindrance. The strong simplification here (also present in De Boer & Perelson, 1994) is to assume that these conjugation sites are independent and equivalent, meaning that conjugation of a T cell in a particular conjugation site does not affect the conjugation of another T cell in an adjacent site. This postulate rules out any possible cooperativity effects (positive or negative) in the process of T cell–APC conjugation. Again, this assumption results in a simpler mathematical model that must be understood as a first approximation to the complexity of the real system. The number of conjugation sites per APC is defined in the model as an integer parameter s, which is varied within a realistic range of values.

(4) The process of formation and dissociation of a conjugate between a T cell and APC can be captured by the same formalism of first-order association reaction of ligand and receptor, leading to a conjugation constant K that is formally analogous to an affinity constant.

The process of T cell–APC conjugation is quite complex involving the concerted expression of many different adhesion molecules and some sort of synchronization of the signalling events in both cells (Dustin & Springer, 1991; Clark

& Ledbetter, 1994). We simplify all these processes assuming that the kinetics of formation of a T cell–APC conjugate is first order in both the concentration of APC conjugation sites (see postulate 3) and the concentration of T cells, and that the kinetics of dissociation of the conjugates is first order in the concentration of conjugates. This leads to an equilibrium constant, formally analogous to the affinity constant in receptor–ligand complex formation, that herein is termed conjugation constant. This terminology is appropriate because it distinguishes the molecular affinity of the TCR for its ligand from the conjugation constant in the interaction between a T cell and its APC.

2.2. A SYSTEM OF ORDINARY DIFFERENTIAL EQUATIONS

The dynamics of populations of cells is described by a generic set of ordinary differential equations (ODE) in the following variables: the total number of regulatory cells R, the total number of effector cells E, the total number of APC A, and the number of multicellular conjugates involving one APC with i effector cells and j regulatory cells A_{ij}.

For E and R cells we have, respectively,

$$\frac{dE}{dt} = \sigma_E + \sum_{i=1}^{s}\sum_{j=0}^{s} \alpha_E(i,j)A_{i,j} - \delta_E E_F, \quad (1)$$

$$\frac{dR}{dt} = \sigma_R + \sum_{j=1}^{s}\sum_{i=0}^{s} \alpha_R(i,j)A_{i,j} - \delta_R R_F, \quad (2)$$

where the involved quantities are defined in Table 1.

The equations for E [eqn (1)] and R [eqn (2)] have three terms. The first term represents the influx of new T cells, which is assumed to be constant. The second term accounts for the dynamical consequences of the interaction of R or E cells with the remaining cells in the system. This term is a linear combination of the conjugates formed by APCs with both classes of T cells, implementing postulate 1. Different settings of the coefficients $\alpha_E(i,j)$ and $\alpha_R(i,j)$, as detailed later are used to account for alternative interaction mechanisms. The third term represents the process of cell death that is assumed to be a simple exponential decay of the free T cells. Note that because only free T cells can die the conjugation with the APC acts as a survival factor.

The variables counting the multicellular conjugates $A_{i,j}$ are only defined when $(i,j) \in (N, N)$ and $(i+j) \leqslant s$ giving rise to $s(s+1)/2 - 1$ equations defined generically as

$$\begin{aligned}\frac{dA_{i,j}}{dt} =& (s-i-j+1)c(A_{i-1,j}E_F + A_{i,j-1}R_F) \\ & -(d_E i + d_R j)A_{i,j} - (s-i-j)c(E_F + R_F)A_{i,j} \\ & + (d_E(i+1)A_{i+1,j} + d_R(j+1)A_{i,j+1}) \quad (3)\end{aligned}$$

and a conservation equation that accounts for postulate 2, and that allows us to drop the differential equation on the non-conjugated APC cells $(A_{0,0})$:

$$A = \sum_{j=0}^{s}\sum_{i=0}^{s} A_{i,j}. \quad (4)$$

The equations for $A_{i,j}$ [defined generically according to eqn (3)] have four terms which represent: (I) the increase in the number of conjugates $A_{i,j}$ formed by the association of free E or R cells with the conjugates of lower order $A_{i-1,j}$ or $A_{i,j-1}$, respectively; (II) the decrease in the number of conjugates $A_{i,j}$ by dissociation of either E or R cells to these conjugates; (III) the decrease in $A_{i,j}$ by association of either free E or R cells to these conjugates; (IV) the increase of the number of conjugates $A_{i,j}$ by dissociation of either E or R cells from the conjugate of order $A_{i+1,j}$ or $A_{i,j+1}$, respectively.

Note that the formulation of eqn (3) is the most synthetic we have found, but it requires an appropriate definition of the quantities $A_{i,j}$ (other than the variables that count the numbers of conjugate in each class (see Table 1), such that whenever the combination i, j is biologically unreasonable its value is set to zero. For example, when $i+j$ is larger than s the corresponding conjugate does not exist and therefore the value of $A_{i,j}$ is set to zero (Table 1). After substituting in eqn (3) the latter type of biological constraints the final system of equations for the conjugates $A_{i,j}$ is formed. Note that in this process some terms are lost in the equations for the conjugates in the extreme part of the system ($(i$ or $j) = 0$ and $(i+j) = s$).

TABLE 1

Symbol	Definition	Biological meaning
Variables		
E	$\in R^+$	Total number of effector T cells
R	$\in R^+$	Total number of regulatory T cells
$A_{i,j}$	$\begin{cases} \equiv 0 & \text{if } (i \text{ or } j) < 0 \\ \in R^+ & \text{if } (i+j) \leqslant s \\ \equiv 0 & \text{if } (i+j) > s \end{cases}$	Number of APC cells conjugated with i E cells and j R, cells
Intermediate variables		
E_c	$= \sum_{i=1}^{s} \sum_{j=0}^{s} iA_{i,j}$	Total number of conjugated E cells
R_c	$= \sum_{i=1}^{s} \sum_{j=0}^{s} jA_{i,j}$	Total number of conjugated R cells
E_F	$= E - E_c$ $= E - \sum_{i=1}^{s} \sum_{j=0}^{s} iA_{i,j}$	Total number of free E cells
R_F	$= R - R_c$ $= R - \sum_{i=1}^{s} \sum_{j=0}^{s} jA_{i,j}$	Total number of free R cells
Parameters		
S	$\in N$	Total number of conjugation sites per APC
σ_E, σ_R	$\in R^+$	Influx of new E and R cells, respectively
δ_E, δ_R	$\in R^+$	Rate constant of the death of free E and R cells, respectively
c	$\in R^+$	Rate constant for the formation of a conjugate between T cells and APC sites
d_E, d_R	$\in R^+$	Rate constants for the dissociation of E or A cells from an APC site
$\alpha_E(i,j)$ $\alpha_R(i,j)$	$\in R$	Interaction coefficients determining the effect on the populations of E or R cells of conjugates of i E cells, j R cells and an APC. Different models can be represented as different sets of these coefficients (see Section 2.3 in text)

2.3. A NON-DIMENSIONAL SYSTEM OF ODES

The ODE system was rendered non-dimensional by dividing the number of cells in each class and the number of conjugates by the total number of APC A, and by substituting real time by the non-dimensional time x, divided by the death rate constant of effector cells δ_E. This yields

$$\frac{de}{dx} = \sigma_e + \sum_{i=1}^{s} \sum_{j=0}^{s} \alpha_e(i,j) a_{i,j} - e_f, \tag{5}$$

$$\theta_r \frac{dr}{dx} = \sigma_r + \sum_{j=1}^{s} \sum_{i=0}^{s} \alpha_r(i,j) a_{i,j} - r_f, \tag{6}$$

$$\theta_a \frac{da_{i,j}}{dx} = (s-i-j+1)K_e(a_{i-1,j}e_f + a_{i,j-1}r_f) - (i+\vartheta j)a_{i,j} - (s-i-j)K_e(e_f + r_f)a_{i,j} + (i+1)a_{i+1,j} + \vartheta(j+1)a_{i,j+1}. \tag{7}$$

The conservation equation (4) is transformed to the normalization condition for the distribution of frequencies a_{ij} (fraction of APCs conjugated with i E cells and j R cells)

$$1 = \sum_{j=0}^{s} \sum_{i=0}^{s} a_{i,j}. \tag{8}$$

The non-dimensional parameters and variables are defined as follows:

$$e = \frac{E}{A}, \quad r = \frac{R}{A}, \quad x = \delta_E t, \quad a_{i,j} = \frac{A_{i,j}}{A},$$

$$e_f = e - \sum_{i=1}^{s}\sum_{j=0}^{s} i a_{i,j}, \quad r_f = r - \sum_{j=1}^{s}\sum_{i=0}^{s} j a_{i,j},$$

$$\sigma_e = \frac{\sigma_E}{\delta_E A}, \quad \sigma_r = \frac{\sigma_R}{\delta_R A}, \quad K_e = \frac{c}{d_E} A, \quad K_r = \frac{c}{d_R} A,$$

$$\alpha_e(i,j) = \frac{\alpha_E(i,j)}{\delta_E}, \quad \alpha_r(i,j) = \frac{\alpha_R(i,j)}{\delta_R}, \quad \vartheta = \frac{K_e}{K_r},$$

$$\theta_r = \frac{\delta_E}{\delta_R}, \quad \theta_a = \frac{\delta_E}{d_E}.$$

2.4. A QUASI-STEADY-STATE APPROXIMATION

The processes of formation and dissociation of conjugates are relatively fast as compared to the overall dynamics of the T cell populations. While a T cell remains conjugated with its APC for a few hours, the T cell mitotic cycle lasts on average 12 hr and the T cell lifespan is at least several days. Under these conditions, it is reasonable to assume that the fraction of conjugates $a_{i,j}$ is in quasi-steady state, reducing the system to two ODEs in the non-dimensional variables e and r:

$$\frac{de}{dx} = \sigma_e + \sum_{i=1}^{s}\sum_{j=0}^{s} \alpha_e(i,j) a_{i,j}(e,r) - e_f, \tag{9}$$

$$\theta_r \frac{dr}{dx} = \sigma_r + \sum_{j=1}^{s}\sum_{i=0}^{s} \alpha_r(i,j) a_{i,j}(e,r) - r_f. \tag{10}$$

The distribution of the conjugates is now defined as

$$1 = \sum_{j=0}^{s}\sum_{i=0}^{s} a_{i,j}(e,r), \tag{11}$$

where $a_{i,j}(e,r)$ is for each pair of values of e and r, the frequency of APCs which are conjugated with i E cells and j R cells, when the processes of formation and dissociation of conjugates reach the equilibrium. In principle, this frequency can be calculated by solving a system of algebraic equations:

$$\begin{aligned} 0 =& (s-i-j+1)K_e(a_{i-1,j}(e,r)e_f + a_{i,j-1}(e,r)r_f) \\ & - (i+\vartheta j)a_{i,j}(e,r) - (s-i-j)K_e(e_f + r_f) \\ & \times a_{i,j}(e,r) + (i+1)a_{i+1,j}(e,r) \\ & + \vartheta(j+1)a_{i,j+1}(e,r). \end{aligned} \tag{12}$$

This system of equations is quadratic in $a_{i,j}(e,r)$, and there is no general analytic solution available for it. The following section proposes an analytic solution for our particular case, based on its particular simplifying properties.

2.5. AN ANALYTIC SOLUTION FOR $a_{i,j}(e,r)$

Taking into account the homogeneity of the APCs and the independence of their conjugation sites, the task of obtaining $a_{i,j}(e,r)$ can be divided into two separate problems. First, to obtain the total number of effector and regulatory cells conjugated with the APC sites, respectively, E_c and R_c, and second to distribute them among the individual APC sites.

The first problem has been addressed before by De Boer and co-workers (De Boer & Perelson, 1994, 1995, 1997), who derived several approximations for the general problem of calculating the number of conjugates formed by n classes of T cells with m classes of APCs. From our own experience none of these approximations is good enough in the problem at stake. Fortunately, in the particular cases of just a few different T cell populations (less than three) it can be solved analytically, as demonstrated in Appendix A. Briefly, the solution is obtained by first computing the total number of free antigenic sites (F) at equilibrium. The latter quantity can be expressed in general as an algebraic equation of order equal to the number of T cells species in the system plus 1. In the particular case of two T cell classes E and R the following third-order equation for non-dimensional variables is derived, where f is now the number of free sites per APC ($f = F/A$):

$$\begin{aligned} 0 =& \{K_e K_r\} f^3 + \{K_e + K_r - K_e K_r(s-r-e)\} f^2 \\ & + \{1 - K_e(s-e) - K_r(s-r)\} f - s. \end{aligned} \tag{13}$$

The three roots for eqn (13), although complicated, can be obtained analytically. Only one is biologically meaningful and is used to obtain the number of conjugated E and R cells per APC in the system according to the following relations:

$$e_c = \frac{fK_e}{1+fK_e}e, \quad r_c = \frac{fK_r}{1+fK_r}r. \qquad (14)$$

The second problem is more complex and hitherto has not been addressed in biological modelling. However, it has some well-known analogues in mathematics and physics. For instance, the work on a lattice gas model (Roman *et al.*, 1996) has given us clues to address it. The desired quasi-steady-state distribution ($a_{i,j}(e, r)$) represents the probability of finding an APC conjugated with iE cells and jR cells, given the total number of APC A, the number of conjugated E cells and the number of conjugated R cells, respectively, E_c and R_c. This quantity can be obtained in two steps, considering the equivalence of conjugated E and R cells. First, we obtain the probability of having an APC with $i+j$ T cells of any kind. And then we multiply it by the probability that within a random sample of $i+j$ T cells, drawn from a population of E_c and R_c R cells, i are E cells and j are R cells. Both steps of the problem are of the same nature corresponding to the classical combinatorial problem of sampling without replacement. The solution to this problem can be found elsewhere (Hoel, 1963) and is expressed in terms of the hyper-geometric distribution. Using the latter analysis the following expression for $a_{i,j}(e, r)$ is obtained:

$$a_{i,j}(e_c, r_c) = Hyp(i+j, (e_c+r_c)A, sA, s) \times Hyp(i, e_cA, (e_c+r_c)A, i+j), \qquad (15)$$

where Hyp is the hypergeometric distribution defined as

$$Hyp(N, N_0, N, L) = \binom{N_0}{N}\binom{M-N_0}{L-N}\Big/\binom{M}{L}. \qquad (16)$$

Equations (15) and (16) allow us to calculate the $a_{i,j}(e, r)$ needed in eqns (9)–(11). This distribution

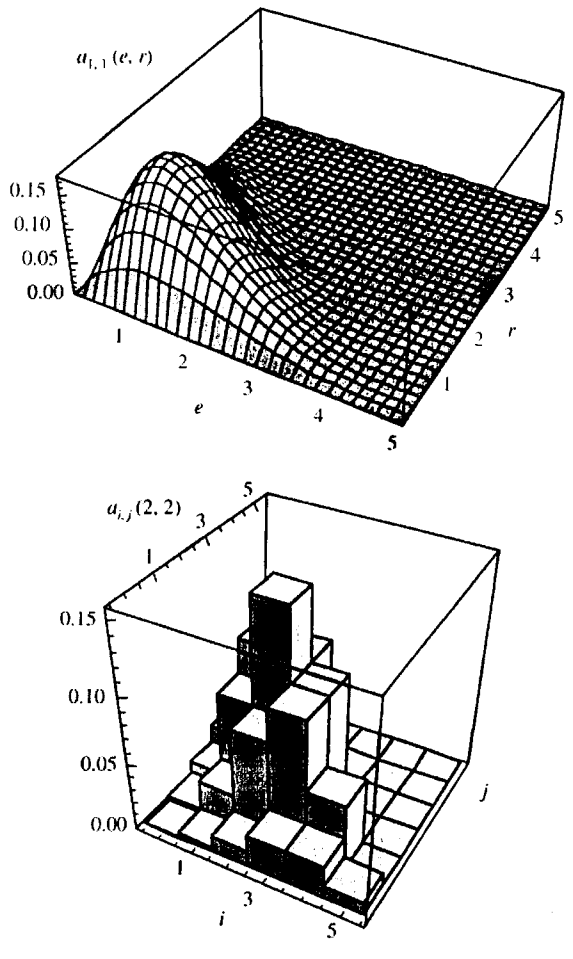

FIG. 1. Distribution of conjugates composed of i effector cells and j regulatory cells when the number of regulatory and effector cells per APC is, respectively, r_e and e_c. See text for details.

is illustrated in Fig. 1. We have confirmed the validity of the latter approach by comparing the expression obtained here and the numeric solution of the set of eqns (12) (data not shown).

2.6. QUALITATIVELY DIFFERENT MECHANISMS OF SUPPRESSION CAN BE MODELLED BY SETTING THE VALUES OF THE PAIRS $\alpha_e(i,j)$ AND $\alpha_r(i,j)$

The basic model developed here for the population dynamics of regulatory and effector T cell populations can be used to represent different putative mechanisms by which regulatory T cells suppress the activation and proliferation of effector T cells. This can be achieved by setting the pair of parameters $\alpha_e(i,j)$ and $\alpha_r(i,j)$ in such a

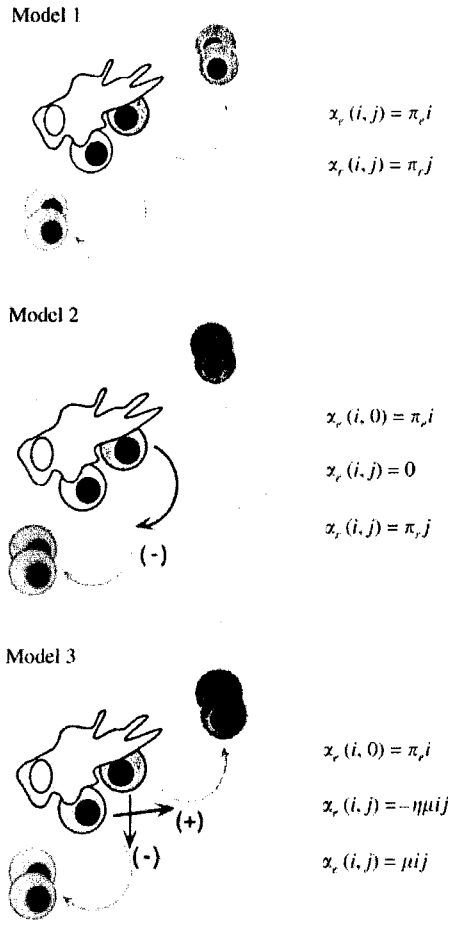

FIG. 2. Cartoon illustrating the different hypothetical mechanisms by which regulatory cells may suppress effector cells while both participate in multicellular conjugates with the APC.

way that they represent the net effect of different processes. For example, the case in which the regulatory cell R prevents the proliferation of the effector cell E when both are conjugated to the same APC can be described by setting $\alpha_e(i,j) < \alpha_e(i,0)$ (with j positive). Similarly, if regulatory cells function by actively killing the effector cells E, this can be implemented by setting $\alpha_e(i,0) > 0$ and $\alpha_e(i,j) < 0$. Using this simple feature of the model in the following sections we will model three basic classes of interaction between regulatory and effector cells (Fig. 2): (1) simple competition for conjugation sites; (2) regulatory cells inhibit the proliferation of effectors cells, but are not affected by the latter (except for competition); (3) the growth of the regulatory cells is dependent on effector cells while the growth of the latter is inhibited by regulatory cells.

One of the main results of this paper is the actual formalism to deal with multicellular conjugates of T cells with APC. In the following sections, we report the results of applying this formalism to model the three candidate mechanisms of interaction between effector and regulatory cells. For each mechanism we formulate a particular model, analyse its phase plane and dependence on parameters. The properties of each model are interpreted and discussed in the context of experimental observations.

3.1. MODEL 1: E AND R CELLS COMPETE FOR CONJUGATION SITES

The first hypothesis concerning the mechanism of T-cell-mediated immune-regulation is competition: R cells are simply cells that are themselves unable to trigger effector function but may interfere with E cells by competing with them for the use of some limited growth factor. This has been made explicit and investigated experimentally by Lechler (Lombardi *et al.*, 1994) and Waldmann (Cobbold *et al.*, 1996), who proposed that anergic T cells could play the role of regulatory cells. Because anergic cells do not produce IL-2, but express high levels of IL-2 receptor they could act as a sink for IL-2, which is required for the growth of normal T cells. In our model, competition between E and R cells for a growth factor stems naturally from the fact that both classes of cells require conjugation with the APC in order to survive or proliferate. The fixed number of conjugation sites acts as a limiting resource (De Boer & Perelson, 1994; Borghans *et al.*, 1999). The equations describing this system are obtained by defining $\alpha_e(i,j) = \pi_e i$ and $\alpha_r(i,j) = \pi_r j$, where π_e and π_r are the ratios between proliferation rate and death rate constants, respectively, for E and R cells. This yields

$$\frac{de}{dx} = \sigma_e + \pi_e e_c - e_f, \tag{17}$$

$$\theta_r \frac{dr}{dx} = \sigma_r + \pi_r r_c - r_f. \tag{18}$$

The quantities e_c and r_c are calculated as described in the previous section. Note that

conjugates $a_{i,j}$ do not appear in these equations because there is no direct interaction amongst the different T cells; the E and R cells can only interfere by occupying the conjugation sites on the APC, giving rise to a competitive interaction which is symmetric.

3.1.1. *Modelling results*

Competition models have been extensively studied in theoretical ecology and more recently in theoretical immunology. It is easier to analyse the model when there is no influx of new cells, i.e. when σ_e and σ_r are set to zero, and then to study the effect of a small influx. This approach will be followed here and in the following sections.

In the absence of influx, the nullclines for e and r in the system are, respectively,

$$e = 0$$

and

$$\frac{1}{\pi_e + 1} e + \frac{1}{(K_e/K_r)\pi_e + 1} r = s - \frac{1}{K_e \pi_e} \tag{19}$$

and

$$r = 0$$

and

$$\frac{1}{(K_r/K_e)\pi_r + 1} e + \frac{1}{\pi_r + 1} r = s - \frac{1}{K_r \pi_r}. \tag{20}$$

Heretofore, we will refer to the nullclines in which the concentrations of r or e are zero or positive, respectively, as the trivial and the non-trivial nullclines. The non-trivial nullclines are two straight lines [eqns (19) and (20)], which do not intersect in positive ranges unless they are identical. The condition for identity is

$$K_r \pi_r = K_e \pi_e. \tag{21}$$

The system has three possible steady states at the intersection between the nullclines in the space (r, e): one trivial steady state (0, 0) and two non-trivial steady states defined as

$$\left(s(\pi_r + 1) - \frac{1}{K_r} - \frac{1}{K_r \pi_r}, 0\right)$$

and

$$\left(0, s(\pi_e + 1) - \frac{1}{K_e} - \frac{1}{K_e \pi_e}\right). \tag{22}$$

The latter are only meaningful [$(e, r) \in (R_0^+, R_0^+)$], respectively, when the following conditions are fulfilled:

$$sK_r \pi_r > 1 \quad \text{and} \quad sK_e \pi_e > 1. \tag{23}$$

Conditions (21) and (23) define three regions in the parameter space $K_e \pi_e$ vs. $K_r \pi_r$ corresponding to three biologically distinct situations (Fig. 3). In region O neither R nor E populations are sustainable, in region I the growth capacity of the effector population is higher than the one of the regulatory population such that the latter is excluded, and in region II the growth capacity of the regulatory population is higher than the one of the effector population and the latter is excluded. Representative phase planes corresponding to regions I and II are illustrated also in Fig. 3.

The basic feature of the model is that, irrespective of the parameter settings, it has always a globally stable equilibrium. If the subpopulations of T cells are sustainable, then only one of them will persist competing out the other one. Which population remains either regulatory or effector cells, depends on the parameter regime.

The algebraic analysis of the system when the source term is positive is quite complicated, but from numerical and graphical analysis it is easy to understand that the qualitative properties of the system remain the same (dashed lines in the phase planes in Fig. 3). Now there are only two nullclines in the system, one for e and one for r. The nullcline of r (or e) is a curve that tends asymptotically to (σ_r(σ_e) when e(r) tends to infinity and tends asymptotically to the nullcline defined by eqn (19) [eqn (20)] when e(r) tends to zero. Under these conditions the trivial and the unstable steady states observed with a null source term, disappear and there is only one equilibrium point, which is stable, in the intersection between the nullclines. The source term changes the numbers or regulatory and effector cells at the equilibrium and also changes slightly the conditions on the parameters separating regions I and II.

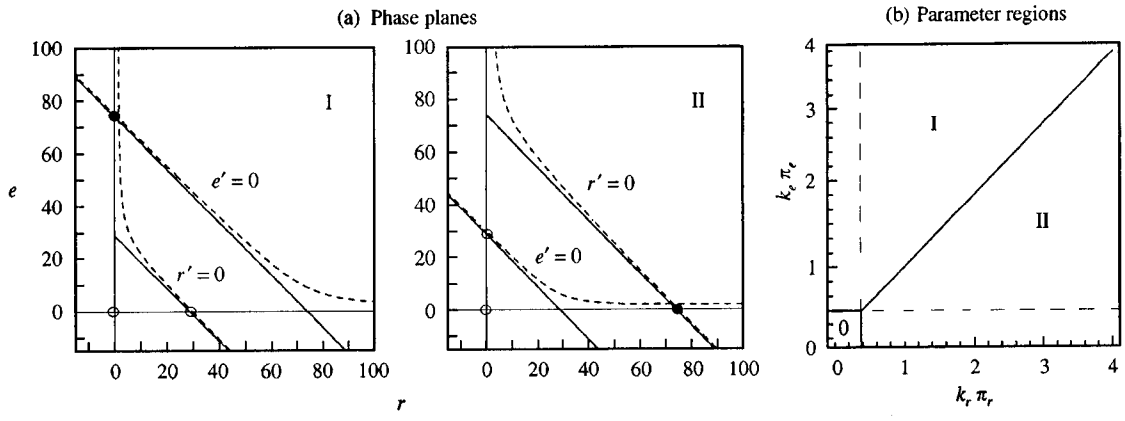

FIG. 3. Phase planes and parameter regions of Model 1 featuring simple competition between regulatory (r) and effector (e) T cell populations. (a) two typical phase planes are illustrated corresponding to regions I and II (in b). In the first, the effector cell population always competitively excludes the regulatory cell population; in the second, the regulatory cell population excludes the effector cell population. The nullclines obtained in the absence (——) and the presence (- - - -) of an influx of R and E cells are depicted. Filled and empty circles represent, respectively, stable and unstable equilibria. The particular parameters are $\pi_e = 14$, $\pi_r = 5$ (a) and $\pi_e = 14$, $\pi_r = 5$ (b); the remaining parameters are $s = 5$, $K_e = K_r = 1$, $\sigma_e = \sigma_r = 0$ or 1. (b) The different parameter regions in the model are depicted in the plane $k_e\pi_e$ vs. $k_r\pi_r$ with $s = 3$ and $\sigma_e = \sigma_r = 0$. There are three parameter regions O, I and II. In region O, the only equilibrium is the trivial (0, 0) (not shown).

3.1.2. *Biological interpretation*

The interpretation of the results of the present model and also in the ones that follow requires an additional assumption on the meaning of the equilibria dominated by either regulatory or effector lymphocytes. As in our previous proposal (Carneiro *et al.*, 1995), we will interpret the equilibrium dominated by regulatory cells as tolerance or unresponsiveness and the state dominated by effectors as an effective immune response or autoimmunity. The rationale behind this assumption is that the T cells themselves do not mediate the effective immune response, but they trigger it.

The major outstanding property of this model is that for any interesting parameter regime there is only a globally stable equilibrium characterized by one of the T cell populations excluding by competition the other one. In general, models with this property can only explain different qualitative responses to antigen such as tolerance and immunity based on an appropriate tuning of parameter values. The change in parameters is of course outside the scope of the model itself and can only be externally controlled. Nevertheless, the possibility that this may happen in the real immune system deserves some discussion. We will consider briefly two hypotheses for how parameters of the population dynamics of effector and regulatory cells could be controlled "externally" in the immune system. Both hypotheses have some bearing on some proposals in the literature.

As a first hypothesis, suppose that regulatory cells have higher affinity for tissue antigen than effector cells ($K_r > K_e$), while for other antigens the affinity of effector cells is higher than the one of regulatory cells ($K_e < K_r$). This situation would allow a simple understanding of tolerance to body tissues and immunity to invading pathogens. The differential affinities in antigen recognition could be controlled by an appropriate selection of the TCR repertoires of regulatory and effector cell populations in the thymus, just as proposed before by several authors (Modigliani *et al.*, 1996b; Itoh *et al.*, 1999).

As to the second hypothesis, suppose that there are two classes of APCs one that will promote preferentially the growth of effector cells and one that will promote the growth of the regulatory cells. Consequently, the nature of antigenic presentation defines whether the steady state will be dominated by either regulatory or effector cells, i.e. whether tolerance or immunity is reached. This second example takes the key decisions out of the T cells themselves and it also provides a potential explanation of tolerance to body

tissues and immunity to invading pathogens. This example could be matched with the ideas of the danger theory of Matzinger and co-workers (Matzinger, 1994).

Although these two hypotheses are immunologically plausible, they are challenged by experimental observations on dominant tolerance. The basic experimental procedure in dominant tolerance is the adoptive transfer into naive recipients of mixtures of purified T cells from tolerant and immune donors, sources, respectively, of regulatory and effector cell populations. The recipients will become either tolerant or immune after reconstitution of their T cell compartment, depending on the initial composition of the inoculum. The only experimental variables in these experiments are the numbers of effector and regulatory cells, which map to the variables in our model. All the other parameters being kept constant, different initial proportions of effector and regulatory cells lead to qualitatively different steady states, making it hard to accept that the system *in vivo* would be globally stable. These considerations render the first hypothesis less straightforward,* and readily reject the second hypothesis.

Overall, any model predicting a globally stable system can be interpreted in terms of dominant tolerance in a non-straightforward way that requires additional assumptions on how parameters are controlled. Moreover, these additional assumptions are controversial or even not supported by experimental observations. These considerations open the way to the next model in which a bistability parameter regime is found, and that leads to a more straightforward interpretation of the results of adoptive transfers.

3.2. MODEL 2: R CELLS INHIBIT THE PROLIFERATION OF E CELLS

The second candidate model assumes that R cells inhibit the proliferation of E cells during simultaneous conjugation with APC. This inhibitory interaction, in contrast to the basic competition for conjugation sites, is asymmetric. This hypothesis is perhaps the most popular in the field of dominant tolerance. It stems from the observation that *in vitro* proliferation of normal T cells is inhibited by anergic/regulatory T cells (Lombardi *et al.*, 1995; Read *et al.*, 1998; Takahashi *et al.*, 1998; Thornton & Shevach, 1998) and that the extent of expansion of donor cells in recipient animals in the presence or absence of regulatory cells is different (Powrie *et al.*, 1997; Annacker *et al.*, 2000). The equations describing this particular mechanism are obtained from the general model by setting $\alpha_e(i,j) = \omega(i,j)\pi_e i$ and $\alpha_r(i,j) = \pi_r j$, where $\omega(i,j)$ is an appropriate function that determines the proliferation rate of E cells as a function of the number of R cells present on the same conjugate. To simplify, it is assumed here that the presence of a single R cell in a conjugate is enough to abrogate the proliferation of all the E cells on the same conjugate. This approximation maximizes the advantage of the R cells over the E cells. Mathematically, we have $\omega(i,j) = 0$ and $\omega(i,0) = 1$ with $j > 1$. The differential equations are:

$$\frac{de}{dx} = \sigma_e + \pi_e \sum_{i=1}^{s} i a_{i,0}(e,r) - e_f, \qquad (24)$$

$$\theta_r \frac{dr}{dx} = \sigma_r + \pi_r r_c - r_f \qquad (25)$$

with (see Appendix B):

$$\sum_{i=1}^{s} i a_{i,0}(e,r) = Hyp(0, r_c A, sA, s) \frac{s}{s - r_c} e_c. \qquad (26)$$

* The first hypothesis is compatible with adoptive transfers of tolerance under the following additional assumptions: regulatory cells are present in T cell population from the tolerant donors; regulatory cells are not present in T cell population from immune donors; and the population dynamics in the recipient animal operates in the parameter regime II, in which regulatory cells always outcompete the effector cells. Under these conditions, the results of adoptive transfers could be interpreted as a limiting dilution of the regulatory cells *in vivo* (Modigliani *et al.*, 1995). At least in some experimental system this interpretation has been ruled out. In these adoptive transfer experiments the number of cells from the tolerant donor in different inocula is kept constant and nevertheless the incidence of tolerance in the recipients decreases as the number of cells from the immune donor is titrated up (Davies *et al.*, 1996b). Also, subpopulations containing effector cells have been isolated from recipients that are tolerant at the steady state (Modigliani *et al.*, 1996b), indicating that they were not competed out by the regulatory cells.

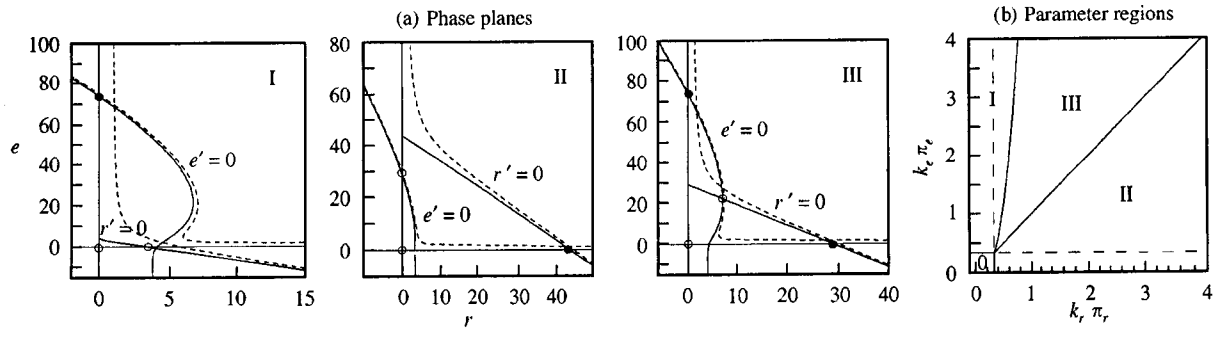

FIG. 4. Phase planes and parameter regions of Model 2 in which regulatory and effector T cell populations compete for conjugation with APC and regulatory cells inhibit the proliferation of effector cells during co-conjugation. (a) Typical phase planes corresponding to parameter regions I, II and III in b. The meaning of the lines and the circles is the same as Fig. 1. Particular parameters: (I) $\pi_e = 14$, $\pi_r = 0.4$; (II) $\pi_e = 8$, $\pi_r = 5$; (III) $\pi_e = 14$, $\pi_r = 1$. The remaining parameters are common: $s = 5$, $K_e = K_r = 1$, $\sigma_e = \sigma_r = 0$ or 1. (b) The parameter regions in the model are defined in the plane $k_e\pi_e$ vs. $k_r\pi_r$ with $s = 3$ and $\sigma_e = \sigma_r = 0$: regions 0, I, II result in globally stable regimes similar to equivalent regions in the model of Fig. 1; the new parameter region III corresponds to a bistable regime (illustrated in A-III).

Note that eqn (25) describing the dynamics of R cells, remains unchanged as compared to eqn (18), because the only way the E cells can interfere with their dynamics is by competition for conjugation sites. Also, note that in the particular case when $s = 1$ this model is reduced to the simple competition as expected from the fact that interactions among T cell populations require at least two cells conjugated with a single APC.

3.2.1. *Modelling results*

The second model is already highly nonlinear due to the presence of the hypergeometric function. It has no general algebraic solution for all the steady states and nullclines as the previous one. To overcome this difficulty, we will resort to numerical phase plane analysis to characterize the possible parameter regions in the model.

As before, we will start the analysis of the model by the case in which the source term is zero, and then generalize the results for the case of a small influx of cells. The non-trivial nullcline for r is the same straight line as in the simple competition model [eqn (20)] (Fig. 4). However, the non-trivial nullcline for e is now a curve, which intersects the previous nullcline [eqn (19)] at $r = 0$ and is always below it for $r > 0$. The deviation of this nullcline from linearity results from the higher sensitivity of the inhibition of the growth of E cells to an increase in the number of R cells (Fig. 4). The curvature of the nullcline for e allows it to intersect the nullcline for r under some parameter regimes, giving rise to the emergence of bistability.

The second model shows four qualitatively different phase planes corresponding to four parameter regions [Fig. 4(b)]. Three of the phase planes, corresponding to regions O, I and II, are comparable to the situations observed in the simple competition model: either both cell populations collapse or there is only a globally stable equilibrium composed of either R or E cells (Fig. 4). However, there is a new parameter region III where the non-trivial nullclines for e and r cross each other such that the system becomes bistable. The corresponding phase plane [Fig. 4(a)] shows four equilibrium points: the trivial equilibrium, the coexistence equilibrium (at the intersection of the non-trivial nullclines), and two equilibria composed exclusively of either R or E cells (at the intersections between non-trivial nullcline of r and trivial nullcline of e and vice versa). The first two equilibria are unstable and the last two are stable. Although the coexistence equilibrium cannot be calculated analytically the equilibria composed of either R or E cells are defined as in eqn (22).

The conditions on the parameters defining region III are the ones that allow persistance of both E and R populations [eqn (23)] and the condition for the intersection of the non-trivial nullclines in the positive values of e and r. They

are given as follows:

$$K_r\pi_r < K_e\pi_e < \frac{1}{sHyp(0, (s - 1/K_r\pi_r)A, sA, s)}. \tag{27}$$

Note that because of the particular shape of the non-trivial nullclines the two conditions in eqn (27) correspond to their intersection at $r = 0$ (left-hand inequality) and their intersection at $e = 0$ (right-hand inequality). These conditions define, respectively, the interfaces between the parametric regions II and III and the parametric regions III and I in Fig. 4(b).

The whole parameter dependence of this model, as well as in the previous one, could be represented in the plane $K_e\pi_e$ vs. $K_r\pi_r$ [Fig. 4(b)] for a given value of the number conjugation sites per APC (s). Increases in the value of s increase the size of the observed region III (data not shown). When s is reduced to 1 region III in the parameter space collapses, as expected from the fact that the model is reduced to a simple competition one.

The major feature of this model as compared to the model of simple competition is that it has a parameter region where bistability exists. Some additional features of interest are related to the effect of the total number of antigen-presenting cells (A). Depending on the initial conditions and the remaining parameters a change in A can have two qualitatively different effects: a bifurcation in which the system switches between phase planes of types I and III; or a change in the relative size of the domains of attraction of the two stable equilibria within the phase plane of type III.

In order to understand the effects of the number of APCs (A) it may be useful to recall that in the non-dimensional system (Section 2.3) the variables e and r are relative to A, and that the parameters K_e and K_r are defined as the product of the conjugation constants of either E or R cells by A. Under these conditions, changes in A alone correspond to change in the coordinates in the space ($K_e\pi_e$ vs. $K_r\pi_r$) along a straight line that passes necessarily through the origin.

With this consideration in mind, it is easy to understand why changes in the number of APCs can lead to a bifurcation between phase planes of types I and III. Any straight line connecting the origin and any point within parameter region III will necessarily pass by region I [Fig. 4(b)]. Note that changes in the number of APCs cannot lead to a bifurcation between regions III and II or between regions II and I. Straight lines connecting the origin and any point in parameter region II do not cross either region I or III. Note also that in the simple competition model, as can be easily inferred from Fig. 3, it is not possible there to switch between regions I and II by changing the number of APC cells.

The second interesting effect of A is on the basins of attraction of the stable equilibria within the bistability regime III (therefore an effect in which no bifurcation is involved). This may be relevant because the final equilibrium reached depends critically on the initial conditions. Thus, relatively high values of A as compared to E + R—low values of $e + r$—will tend to result in the equilibrium dominated by E cells, while relatively low values of A—high values of $e + r$—tend to lead to the equilibrium dominated by R cells (Fig. 5). This result is systematically observed independently of the initial proportions of R and E cells, as long as both populations of cells are initially not null.

As in the previous case, the introduction of a small source term of E and R cells leads to the disappearance of the trivial nullclines. The new resulting nullclines are illustrated as dashed lines in the phase planes of Fig. 4(a) and (b). Graphical and numerical analysis (not shown) indicates that all the properties pointed out here are generic except for the existence of the trivial state (0, 0), which is not present when there is a source term.

The results discussed in this section are systematically observed in models in which the effector cells can only interfere with the growth of regulator by competition for conjugation sites on the APC, but regulatory cells have an extra inhibitory term which reduces the rate of proliferation of the effector cells (data not shown). Mathematically, this is obtained by setting $\omega(i, j) < 1.0$ with $j > 0$ and $\omega(i, 0) = 1.0$. Moreover, when the value $\omega(i, j)$ is negative then the model accommodates a biologically distinct mechanism according to which regulatory cells actively kill the effector cells.

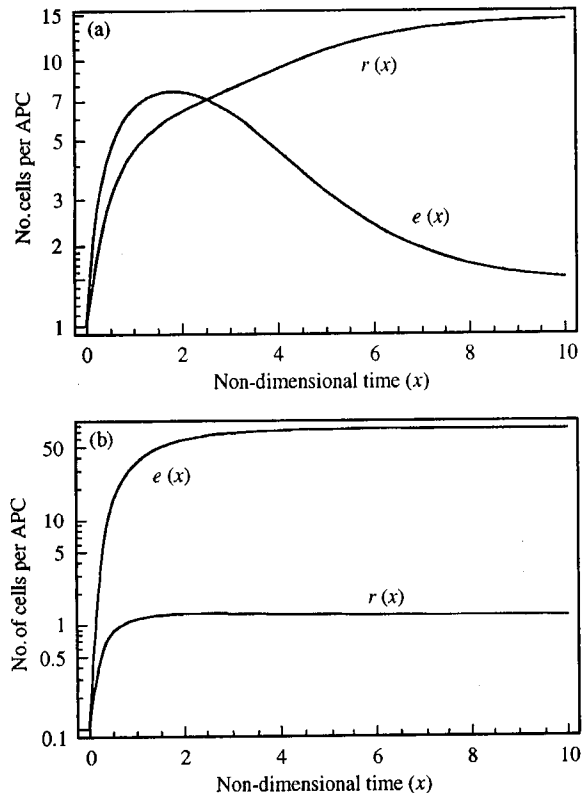

FIG. 5. Effect of changing the number of APC A within the bistability regime III in Model 2. (a) and (b) are time plots of the number of E cells and R cells per APC (e and r, respectively), in two parameter sets within region III differing only in A. For fixed initial conditions a relatively low number of APCs ($A = 1000$) leads to dominance of regulatory cells (a) and a relatively high number of APCs ($A = 10000$) leads to dominance of effector cells (b). Initial conditions: $e(0) = r(0) = 1$. Parameter settings: $\pi_e = 14$, $\pi_r = 5$, $s = 5$, $K_e = K_r = 1$, $\sigma_e = \sigma_r = 1$.

3.2.2. *Biological interpretation*

Again we consider here that the state dominated by regulatory cells is tolerance and the state dominated by effector corresponds to (auto) immunity.

The major property of this model is the existence of parameter region III where bistability is possible. Operating in this region the model may overcome the difficulties discussed in the previous section for models with a globally stable equilibrium. Nevertheless, studies *in vitro* suggest that regulatory cells when stimulated with APCs in the absence of effector cells do not proliferate (Lombardi *et al.*, 1995; Read *et al.*, 1998; Suri-Payer *et al.*, 1998; Takahashi *et al.*, 1998). This observation raises a problem for the present model. Hence, if the proliferation rate of the regulatory population is set to zero then we will be in a region of the parameter space in which effector cells will always dominate [region I in Fig. 4(b)], i.e. we will be in the presence of global stable equilibrium. Therefore, the present model, as the one before, may be inconsistent with the phenomenon of dominant tolerance when the expansion of the regulatory population alone is experimentally undetectable.

Another property that is relevant for the biology of dominant tolerance is the predicted influence of the number of APCs on the basins of attraction of the steady states of the system. According to this model it is always possible to switch from a state dominated by regulatory cells to a system dominated by effector cells (and vice versa) by increasing the number of antigen-presenting cells. The latter tendency has been shown by *in vitro* titration of APCs by both Sakaguchi (Takahashi *et al.*, 1998) and Lechler (Lombardi *et al.*, 1994). Also, it is well known that adoptive transfers of tolerance may fail if the total number of cells in the inoculum is low (Le Douarin *et al.*, 1996; Modigliani *et al.*, 1996b). Typically, this is interpreted as the result of the absence of regulatory cells in the inoculum. The present model suggests an alternative interpretation according to which what is being done in those experiments is to decrease the values of e and r, such that the dominance of effector cells is favoured. This interpretation also provides a straightforward account for the well-documented observation that partial depletion of a normal T cell compartment may lead to autoimmunity (Sakaguchi *et al.*, 1989, 1994). Also, it is interesting to recall here that autoreactivity and a reconsideration of horror autotoxicus was only possible after the discovery of adjuvants by Freund. Different animal models of autoimmunity were discovered by injecting self-antigens in CFA, the most notable example being experimental allergic encephalomyelitis (EAE). One of the effects of adjuvants is simply promoting the influx of antigen-presenting cells to the place where the antigen is and their local activation. The present model, but also the one discussed in the next section, gives a natural interpretation to this well-known fact.

3.3. MODEL 3: THE GROWTH OF R CELLS IS DEPENDENT ON E CELLS

The third particular case of the general model encloses in a single mathematical formalism several different mechanisms of interest. It is obtained by setting $\alpha_e(i,j) = \omega(i,j)\pi_e i - \eta\mu ij$ and $\alpha_r(i,j) = \pi_r j + \mu ij$ in eqns (9)–(11), where $\omega(i,j)$ is defined as in the previous section. This yields

$$\frac{de}{dx} = \sigma_e + \pi_e \sum_{i=1}^{s} i a_{i,0}(e,r) - \eta\mu \sum_{i=1}^{s}\sum_{j=1}^{s} ij a_{i,j}(e,r) - e_f, \tag{28}$$

$$\theta_r \frac{dr}{dx} = \sigma_r + \pi_r r_e + \mu \sum_{i=1}^{s}\sum_{j=1}^{s} ij a_{i,j}(e,r) - r_f, \tag{29}$$

where we have (see demonstration in Appendix B)

$$\sum_{i=1}^{s}\sum_{j=1}^{s} ij a_{i,j}(e,r) = \frac{(s-1)A}{(sA-1)} e_c r_c \approx \frac{s-1}{s} e_c r_c. \tag{30}$$

The latter is a good approximation for values of A big enough. In the following analysis of this model, values of A greater than 100 are systematically used (even when studying the dependence on A).

From the comparison of eqns (28) and (29) and eqns (24)–(26), it is noticeable that this models adds a new interaction term to the previous one (being reduced to it when $\mu = 0$). In order to explore the properties that are unique to this new term of interaction we will set π_r to zero, which will naturally account for the observation that regulatory cells do not proliferate when cultivated alone with APC.

The new interaction can be interpreted in different ways depending on the values of μ and η. Two particular cases with biological interest are the following. In the case $\eta = 0$ the new interaction term is understood as an effector-dependent proliferation of the regulatory cells. In the case $\eta = 1/\theta_r$ [note that the relationship between the time-scales in eqns (28) and (29) is given by θ_r] the equations represent a regulatory-dependent differention of effector to regulatory cells, as it has been proposed by Waldmann *et al.*, (Qin *et al.*, 1993) and others (Modigliani *et al.*, 1995). The same parameter setting would also account for the possibility that regulatory cells actually kill the effector cells but are stimulated to proliferate during this interaction. Any other combination of the values of μ and η can of course be interpreted as a mixture of these elementary mechanisms.

3.3.1. *Modelling results*

As in the previous sections, we are going to first analyse the model in the simplest situation where the influx of new T cells is zero and only then generalize the major qualitative properties to the situation when this influx is positive but small.

In this last particular model, in the absence of influx, the non-trivial nullcline for e is similar to the one in the previous section; however, the non-trivial nullcline for r is different, reflecting the effector-dependent growth of R cells (Fig. 6). The latter is a parabolic-curve that never crosses the trivial nullcline of e, i.e. both arms of the curve cross the trivial nullcline of r (Fig. 6). The shape of this nullcline reflects the most distinctive property of the model, which is the impossibility of sustaining an R cell population in the absence of E cells (a direct consequence of setting π_r to zero).

The model shows four types of phase planes in $(e,r) \in (R_0^+, R_0^+)$, depending on how the parameters determine the positions of the nullclines and their intersections (Fig. 6(b)]. As we will pinpoint, they show some properties which are analogous to the ones of the previous models. For this reason we will call them again as parameter regions O, I, II and III. Nevertheless, there are some different properties that will also be indicated.

In the parameter regime O, the phase plane is identical to the ones in all previous models. It is characterized by the presence of only the trivial nullclines in the quadrant (R_0^+, R_0^+), such that the only steady state is the trivial one (0, 0).

The phase plane in parameter region I is characterized by the non-trivial nullcline of e passing in the quadrant (R_0^+, R_0^+) where it does not intersect the non-trivial nullcline of r (whether or not this one is in the same quadrant). Under these conditions the model has one unstable trivial steady state (0, 0) and one globally stable steady state defined by the intersection of the non-trivial nullcline of e and the trivial nullcline of r (Fig. 6). The qualitative properties in this parameter

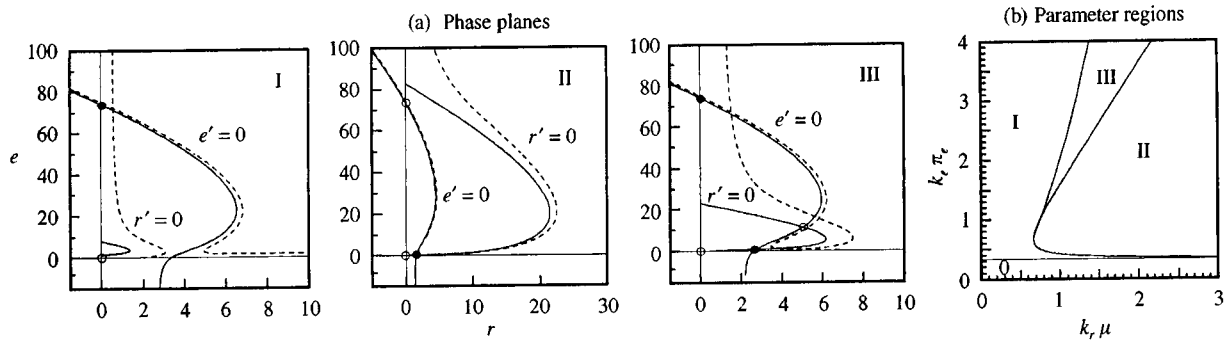

FIG. 6. Phase planes and parameter regions of Model 3 in which regulatory and effector T cell populations compete for conjugation with APC and the growth of the regulatory cell population is dependent on co-conjugation with effector cells. (a) Typical phase planes corresponding to parameter regions I, II and III in b. The meaning of the lines and the circles is the same as in Fig. 1. The grey circle indicates that the focus can be either stable or unstable depending on the parameters. Parameters: $\pi_e = 14$, $\eta = 1$, $s = 5$, $K_e = K_r = 1$, $\sigma_e = \sigma_r = 0$ or 1, and $\mu = 0.3$ (I), $\mu = 4$ (II) and $\mu = 1$ (III). (b) The four possible parameter regions in the model are depicted in the plane $k_e\pi_e$ vs. $k_r d_c$ with $s = 3$ and $\sigma_e = \sigma_r = 0$: regions 0, I, II result in globally stable regimes similar to equivalent regions in the models of Figs 1 and 2; the parameter region III corresponds to a bistable regime as in the model of Fig. 2.

region are therefore very similar to the ones of the previous models in analogous region, namely by the fact that independently of the initial conditions the population of effector cells will always exclude by competition the population of regulatory cells R.

In parameter region II, the non-trivial nullclines of r and e are both in the quadrant $(e, r) \in (R_0^+, R_0^+)$ where they cross only once. Under these conditions there is an additional steady state in the intersection between the two non-trivial nullclines. This is a focus, that is globally stable, because the steady state that was stable in parameter region I, now is unstable. Similar to parameter region II in the previous models, a globally stable steady state is predicted where regulatory cell population persists. However, while in the previous models this steady state was characterized by the exclusion or extinction of the effector cell populations, in this final model, the steady state in which regulatory cells can dominate requires also the persistence of effector cells.

In the phase plane corresponding to parameter region III the non-trivial nullclines of r and e intersect twice, such that a fourth steady state appears. This fourth steady state is a saddle point. In this parameter regime, the steady state dominated by e cells is again stable as in region I, and the focus, where r cells persist, which was always stable in region II, can now be stable or unstable depending on the value of η. When $\eta = 0$ the focus is always stable and when $\eta > 0$ it can be either stable or unstable. This parameter region is similar to region III in the previous model because it displays four steady states and can show bistability. It has, however, two major differences that were already mentioned. First, the focus can actually be unstable such that bistability is not observed (see Fig. 7), and second, when the focus is stable both regulatory and effector cell populations persist.

Although this final model is extremely nonlinear it is interesting that parameter regimes O, I, II and III can be simply represented in the plane $K_e\pi_e$ vs. $K_r\mu$, for different values of s [Fig. 4(b)] and for different ratios $\eta' = \eta K_e/K_r$ [Fig. 6(c)]. The boundaries between region II and regions I and III can be derived analytically as in the case of the previous models, being defined by the following conditions on the parameters:

$$s(K_e\pi_e)^2 - s(s-1)(K_e\pi_e)(K_r\mu) + (s-1)(K_r\mu) = 0. \tag{31}$$

These conditions correspond, respectively to, on the one hand, the intersection of the lower arm of the non-trivial nullcline of r, to the upper arm of the non-trivial nullcline of e at the axis $r = 0$, and on the other hand, the intersection of the upper arm of the non-trivial nullcline of r, to the upper

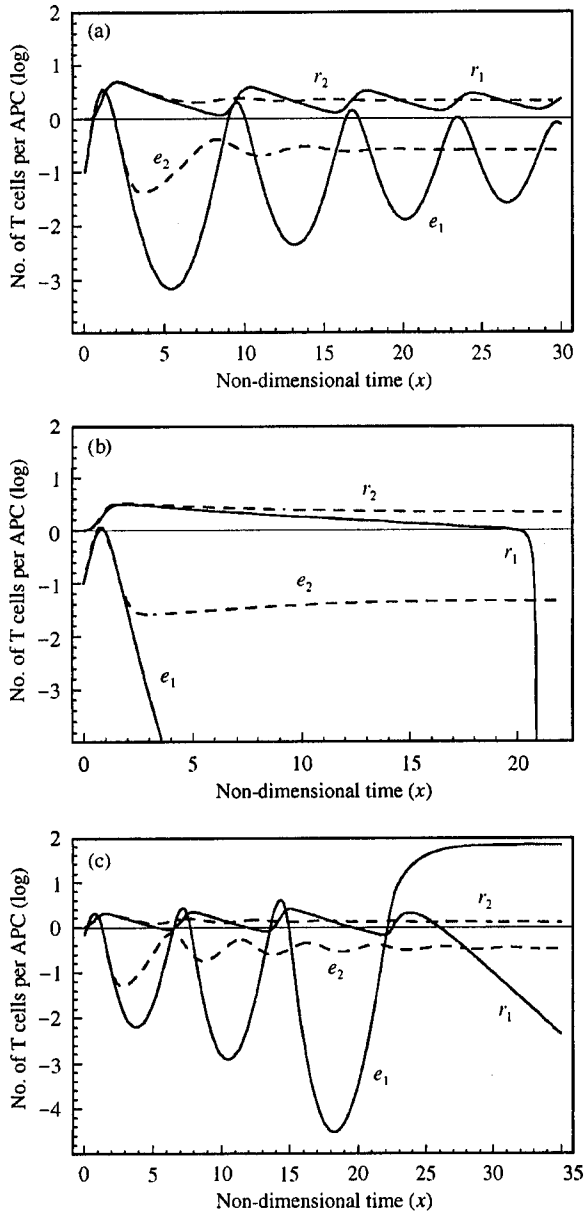

FIG. 7. In Model 3 the influx of new cells to the effector cell population stabilizes the steady state dominated by regulatory cells, within parameter region III. In the absence of an influx of effector cells ($\sigma_e = 0.0$) the dynamics of the system (——) can range from dumped oscillations with low amplitude (a), to dumped oscillations with unrealistic amplitude (b) or even divergent oscillations are obtained, when the equilibrium is unstable (c). A small influx ($\sigma_e = 0.1$) into the effector cell populations stabilizes the dynamics for the system in all these cases (- - - -). Initial conditions: (a) $r(0) = 1$, $e(0) = 0.1$; (b) $r(0) = 1$, $e(0) = 0.1$; (c) $r(0) = 1$, $e(0) = 0.7$. Parameters: (a) $\pi_e = 14$, $\eta = 1$, $\mu = 2$, $s = 5$, $K_e = K_r = 1$, $\sigma_r = 0$; (b) $\pi_e = 14$, $\eta = 1$, $\mu = 2$, $s = 5$, $K_e = K_r = 5$, $\sigma_r = 0$; (c) $\pi_e = 14$, $\eta = 5$, $\mu = 6$, $s = 5$, $K_e = K_r = 1$, $\sigma_r = 0$.

arm of the non-trivial nullcline of e at the axis $r = 0$. As to the boundary between regions I and III we were unable to derive it analytically. The curve in Fig. 6(b) was derived by computing numerically the parameters defining the intersection between the two non-trivial nullclines in the positive quadrant.

While changing η' does not affect the existence of the four regions in the plane $K_e\pi_e$ vs. $K_r\mu$, when s is unitary both regions II and III collapse. This is obviously expected from the assumption that growth of the regulatory cell population is dependent on interactions between effector cells and regulatory cell in multicellular conjugates.

Despite the structural differences in the phase planes of this model and the previous one, they show some similarities: both models can show the same three types of phase planes/parameter regimes (two globally stable regimes dominated by either effector cells or regulatory cell, and bistability with dominance of either regulatory or effector cells); both models show a bifurcation which allows to switch the system between parametric regions III and I by changing parameter A alone; and finally within bistable regime III it is always possible to favour the dominance of E cells by increasing A (reciprocally to favour the dominance of R cells by decreasing A).

As in the previous cases, the addition of a small source term for effector and regulatory cells, does not change the qualitative properties of the model. The only exception is that the introduction of a source term of effector cells but not of regulatory cells is sufficient to stabilize the equilibrium point dominated by regulatory cells (dashed lines Fig. 7). This stabilization is related to an introduction of a curvature in the nullclines crossing in the equilibrium dominated by regulatory cells. As discussed next, this may be a feature which is highly relevant immunologically.

3.3.2. *Biological interpretation*

The model studied in this section displays bistability and essentially the same qualitative behaviour as the one discussed in the previous section: it accounts for titration of tolerance by titration of regulatory cells, for the influence of antigen-presenting cells, etc. In addition, this final model naturally accommodates the observation

that regulatory cells do not proliferate *in vitro* when cultivated alone in the presence of APCs (Lombardi *et al.*, 1994; Read *et al.*, 1998; Takahashi *et al.*, 1998; Thornton & Shevach, 1998), even within the bistability regime. While this observation was, as we mentioned, problematic for the previous model, it is a prerequisite of this final model, which assumes that the expansion of the population of regulatory cells is dependent on the presence of effector cells.

The other property of this last model that is relevant for a comparison with experimental data is the coexistence of both effector and regulatory cells in a state dominated by regulatory cells. This is only predicted by this last model and was not present in any of the previous ones. Experimental reports indicate that populations of both regulatory and effector cells coexist in tolerant animals as can be revealed by selective induction of autoimmunity or tolerance in recipient animals receiving different subpopulations of cells. Many of these experiments are performed in euthymic animals where a residual population of effector cells could be maintained by a continuous influx of thymic-derived cells. All candidate mechanisms that we specified and analysed here are qualitatively compatible with this observation. However, in some cases, the same observation has been made in athymic animals (Modigliani *et al.*, 1996b), and this will strongly favour the mechanisms in which the growth of the regulatory cells population is dependent on continuous interactions with effector cells.

Also interesting from the point of view of the relationship with experimental observations is the finding that the influx of cells can determine the robustness and the stability of the steady state dominated by regulatory cells. Interestingly, it is well known that thymectomized animals, as compared to euthymic animals, are more susceptible to procedures that induce autoimmunity (irradiation, stimulation, etc.). The traditional interpretation is that the maintenance of peripheral tolerance is dependent on a continuous influx of new regulatory cells from the thymus. Interestingly, our results suggest a less intuitive interpretation. Thymectomy would deprive the periphery of new coming effector cells switching the system from a parameter regime in which the state dominated by regulatory cell is stable to a regime in which this state is now unstable or less resistant to perturbations. That is to say, it is the influx of new effector cells from the thymus which reinforces the stability of the tolerant state and not the influx of regulatory cells.

4. Discussion

The goal of the present article was to further our understanding on the mechanisms of linked suppression and its putative role in dominant tolerance, by exploring its requirement of a simultaneous interaction between three cell types. To achieve this goal we developed a mathematical tool to follow the interactions in multicellular conjugates. To our knowledge, this report is the first mathematical analysis of this type of cell interaction.

The dynamical models that take into account the interaction between more than three cell types must *a priori* be quite complex. As we have seen, the number of variables and differential equations, which count the number of high-order conjugates being formed and dissociated, is controlled by the number of conjugation sites on the APC, s. Actually, the number of equations increases with the square of s. The quasi-steady-state approach we followed overcomes this difficulty proposing an algebraic solution for the quasi-steady-state distribution of the high-order conjugates between two classes of T cells and their APC. This allowed us to reduce the number of equations to the actual number of cell types. The strategy involves two steps: the calculation of the number of T cells in the conjugates and their distribution by the APCs. This approach will hold as long as the conjugation sites are independent. Moreover, it can be generalized for more than two T cell subpopulations. However, there is an analytic solution only for systems with up to three subpopulations, since the roots of a polynomium with order equal to the number of subpopulations plus I are involved in calculating the number of cells in each class that are conjugated. For orders greater than 3 some other approximation can be used (De Boer & Perelson, 1995); however, this should be systematically compared with numerical solutions.

The basic model we proposed may represent a very general tool to deal with simultaneous

interactions between more than two cell types since different detailed mechanisms can be specified by an appropriate setting of the interaction coefficients determining the consequences of each high-order conjugate (Table 1). The application of this formal tool in situations other than the current application in dominant tolerance can be quite straightforward. For example, it was proposed recently that the interaction between CD4+ T cells and CTLs does not involve the simultaneous interactions between the two cells on the same antigen-presenting cell, as classically proposed (Mitchison & O'Malley, 1987; Mitchison, 1990), but that the APC may act as a temporal bridge (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). The formalism presented here can be very easily adapted to analyse in detail the quantitative implications of the two different mechanisms (Leon & Carneiro, unpublished data).

An important question at this point is of course: how do these mathematical models help in understanding dominant tolerance mediated by linked-suppression? The experimental systems that reveal and investigate the mechanism of dominant tolerance are very diverse. Many experiments are performed in normal animals while others make use of experimentally manipulated animals. The latter always raise the possibility that we are dealing with some consequence of the manipulation rather than a natural mechanism. In adoptive transfers of tolerance different authors use different T cell subpopulations as sources of effector and regulatory T cells. For example, as source of effector and regulatory cells, respectively. Powrie (Powrie *et al.*, 1997) typically uses CD45RBhigh and low CD4+ T cells while Sakaguchi (Sakaguchi *et al.*, 1995) uses CD25− and CD25+ CD4+ T cells. Although, in general, both CD25+ and CD45Rb low cells are considered to be "antigen experienced", the relationship between the two populations is not straightforward (Read *et al.*, 1998). Under these conditions, it is very difficult to provide a unifying hypothesis. Instead, we illustrated how the general model, made particular for specific cases, can be useful to interpret experimental observations and to narrow down the alternative candidate explanations. We evoked different experimental observations and tried to indicate the conditions under which they can be consistent with the alternative mechanisms of linked suppression. Overall, the modelling results reported here and the whole set of observations that we discussed would strongly favour two candidate mechanisms. These are the ones that are translated by the final model: regulatory T cells inhibit the proliferation of effector cells while they are nevertheless dependent on a growth factor that the latter produce; or regulatory cells inhibit the expansion of the population of effector cells because they convert them to the regulatory phenotype. Either mechanism or both may be operative *in vivo*, maybe even dependent on the life history of the effector cells. Modigliani *et al.* (1996b) actually demonstrated that regulatory cells from thymic epithelium chimeras can both suppress the responses of mature effector cells and promote the differentiation of recent thymic emmigrants into the regulatory phenotype.

The experimental reports on dominant tolerance *in vivo* are most of the time qualitative rather than quantitative. For this reason, this paper explored the generic qualitative properties of the different models. *In vitro* suppression assays are more quantitative than *in vivo* adoptive transfers, and therefore they represent a better area to try to test the quantitative predictions of the models. Elsewhere, we analyse the experimental data of linked suppression *in vitro*. We explore the simple fact that there must be a limit in the frequency of encounters leading to multicellular conjugates, and show that only a few mechanisms could lead to the levels of *in vitro* suppression reported (Leon *et al.*, unpublished data).

The full understanding of dominant tolerance may eventually require a consideration of the clonal diversity of the T cell repertoire. Lafaille *et al.* (1994), and Olivares-Villagomez *et al.* (1998) reported that the onset of autoimmunity mediated by an auto-reactive transgenic population of T cells is prevented by regulatory T cells with an endogenous TCR. A consideration of this type of phenomenon suggests an immediate extension of our present model to consider the dynamics and potential heterogeneity of the population of APCs and clonal composition of regulatory and effector T cell populations. This extension is not only experimentally motivated but also has an important theoretical justification. Hence,

the most serious theoretical problem faced by any candidate model of dominant tolerance is to explain how an organism is able to mount effective immune responses to invading pathogens and remains, nevertheless, self-tolerant (Carneiro, 1997). Although some qualitative considerations about the role of repertoire diversity in tolerance can be found in the literature, the issue has never been addressed properly, and therefore represents an area for development of the modelling strategy reported here.

We thank Jose Faro and João Sousa for many useful discussions, during the realization of this work and John Stewart for critically reading this manuscript.

This work is supported by Program Praxis XXI of the Ministério para Ciência e Tecnologia, Portugal (grant Praxis/P/BIA/10094/1998). JC is supported by Fundação para a Ciência e Tecnologia—Program Praxis XXI (fellowship BPD/11789/97). KL is partially financed by the Fundação Calouste Gulbenkian.

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APPENDIX A

A third-order equation for obtaining the total number of conjugated R and E cells

The number of effector and regulatory cells conjugated with APC, respectively, E_c and R_c, the number of free effector and regulatory cells, respectively, E_F and R_F and the number of free sites F at equilibrium fulfill the following conditions:

$$K_E = \frac{E_C}{FE_F}, \qquad K_R = \frac{R_C}{FR_F}. \tag{A.1}$$

Taking into account the conservation of total T cells (A.2) and total conjugation sites (A.3)

$$E_F = E - E_C, \quad R_F = R - R_C, \tag{A.2}$$

$$F = (sA - E_C - R_C). \tag{A.3}$$

Combining eqns (A.2) and (A.1), we obtain two expressions for F in terms of E_c and E or R_c and R:

$$F = \frac{E_C}{K_E(E - E_C)}, \qquad F = \frac{R_C}{K_R(R - R_C)}. \tag{A.4}$$

Rearranging eqn (A.4) we express E_c and R_c in terms of F and the total number of cells E and R:

$$E_C = \frac{FK_E}{1 + FK_E}E, \qquad R_C = \frac{FK_R}{1 + FK_R}R. \tag{A.5}$$

To obtain E_c and R_c as a function of the number of E and R cells and their respective conjugation constants we first solve for F. Substituting E_c and R_c as defined by eqn (A.5) into eqn (A.3), and rearranging we obtain the following third-order algebraic equation:

$$\begin{aligned} 0 = &\{K_E K_R\}F^3 + \{K_E + K_R - K_E K_R \\ &\times (sA - R - E)\}F^2 + \{1 - K_E(sA - E) \\ &- K_R(sA - R)\}F - sA. \end{aligned} \tag{A.6}$$

This equation has three roots, but only one is biologically meaningful. Although this root of eqn (A.6) can be expressed algebraically it is very complex and will not be presented here. E_c and R_c are then obtained by replacing F in eqns (A.5) by the root of eqn (A.6), and E_F and R_F are obtained by replacing E_c and R_c in eqns (A.3).

We demonstrate that there is only one biologically reasonable root of eqn (A.6) by reduction to the absurd. Assume that there are two roots of F which are biologically reasonable denoted by $F1$ and $F2$. Assume that $F1 > F2$. If the latter is true then eqn (A.3) implies

$$(E_c 2 + R_c 2) > (E_c 1 + R_c 1). \tag{A.7}$$

On the other hand, by using eqn (A.5) the following relationship is derived between the value of $(E_c + R_c)$ and the corresponding value of F.

$$E_C + R_C = \frac{FK_E}{1 + FK_E}E + \frac{FK_R}{1 + FK_R}R. \tag{A.8}$$

This relationship expresses a monotonously increasing dependence between F and its corresponding value of $(E_c + R_c)$, since both belong to R_0^+. The bigger the F the bigger the corresponding $(E_c + R_c)$. So if inequality (A.7) and relation (A.8) are true then necessarily $F2 > F1$. The latter is the negation of the original premise $F1 > F2$ demonstrating the impossibility of having two different biologically reasonable solutions for this equilibrium.

The procedure described here to obtain the equilibrium numbers of APC-conjugated and free calls in a mixture of several T cell populations is easily extended to more than two populations, eventually N of them. In this case, the equation for F is obtained as an algebraic equation of order equal to $N - 1$.

APPENDIX B

Analysing the properties of the distribution of T cells in APC sites

In Section 2.1, it was shown that if the antigenic sites in a single APC are assumed to be independent (postulate 4), then the frequency of APC containing iE cells and jR cells, is given by eqn (15). The latter equation is composed of two hypergeometric distributions defined according to eqn (16). Substituting eqn (16) into eqn (15) and simplifying it yields:

$$a_{i,j,k}(e,r,h) = \frac{\binom{e_C A}{i}\binom{r_C A}{j}\binom{(s-e_C-r_C)A}{s-i-j}}{\binom{sA}{s}}. \tag{B.1}$$

Here it is important to note the symmetry in the latter expression in terms of the E and the R cells, which must be expected, from the symmetry of the problem itself. Note additionally that $(s-e_c+r_c)$ and $s-i+j$ [central elements in eqn (B.1)] are, respectively, the total fraction of free sites per APC and the number of free sites in APC containing iE and jR cells. This gives us a straightforward procedure to extend expression (B.1) to the case of more than two types of T cells. For instance, for three types of cells E, R, H the following is obtained:

$$a_{i,j,k}(e,r,h) = \frac{\binom{e_C A}{i}\binom{r_C A}{j}\binom{h_C A}{k}\binom{(s-e_C-r_C-h_C)A}{s-i-j-k}}{\binom{sA}{s}}. \tag{B.2}$$

Let us study now some simplifications of interest in expression (B.1). Particularly, we are interested in the following relation:

$$\sum_{i=0}^{s-j} i a_{i,j}(e,r) = Hyp[j, r_C A, sA, s]\,\frac{s-j}{s-r_C}\,e_C. \tag{B.3}$$

Note that the particular case of this expression, when $j=0$ is used in Section 3.2. To demonstrate that eqn (B.3) holds it is enough to multiply and divide eqn (B.1) by

$$\binom{(s-r_C)A}{s-j}$$

obtaining:

$$a_{i,j}(e,r) = \frac{\binom{r_C A}{j}\binom{s-r_C)A}{s-j}}{\binom{sA}{s}} \times \frac{\binom{e_C A}{i}\binom{(s-e_C-r_C)A}{s-i-j}}{\binom{(s-r_C)A}{s-j}},$$

which according to eqn (15) is equivalent to

$$a_{i,j}(e,r) = Hyp[j, r_C A, sA, s] \times Hyp[i, e_C A, (s-r_C)A, s-j]. \tag{B.4}$$

Substituting then eqn (B.4) on the left-hand side of eqn (B.3) we get

$$\sum_{i}^{s-j} i a_{i,j}(e,r) = Hyp[j, r_C A, sA, s] \sum_{i=0}^{s-j} i\, Hyp[i, e_C A, (s-r_C)A, s-j]. \tag{B.5}$$

Inserting into eqn (B.5) the expression for the value of the median in hypergeometric distribution reported elsewhere:

$$\langle N\rangle = \sum_{N=0}^{K} N\, Hyp[N, No, M, K] = NoK/M. \tag{B.6}$$

We get the expression proposed in eqn (B.3), which for the particular case $j=0$ stands

$$\sum_{i=0}^{s} i a_{i,0}(e,r) = \frac{Hyp[0, r_B A, sA, s]s}{s-r_C}\,e_C \tag{B.7}$$

Note that the latter expression has a very simple interpretation: the numerator is the number of free sites in APC with $j = 0$ R cells, while the denominator is the total free sites that can be occupied by the E cells "once the conjugated R cells are already distributed".

The other particular relation of interest for us in this work is

$$\sum_{j=0}^{s} \sum_{i=0}^{s-j} ija_{i,j}(e, r) = \frac{(s-1)A}{(sA-1)} e_C r_C. \quad \text{(B.8)}$$

To demonstrate this relation let us first substitute eqn (B.3) on the left-hand side of eqn (B.7) getting

$$H = \sum_{j=0}^{s} j \sum_{i=0}^{s-j} ia_{i,j}(e, r)$$

$$= \sum_{j=0}^{s} Hyp[j, r_C A, sA, s] \frac{s-j}{s-r_C} e_C$$

$$= \sum_{j=0}^{s} j\, Hyp[j, r_C A, sA, s] \frac{s}{s-r_C} e_C$$

$$- \sum_{j=0}^{s} j^2\, Hyp[j, r_C A, sA, s] \frac{1}{s-r_C} e_C. \quad \text{(B.9)}$$

Using expression (B.6) here we get

$$H = s \frac{r_C e_C}{s-r_C} - \frac{e_C}{s-r_C} \sum_{j=0}^{s} j^2\, Hyp[j, r_C A, sA, s]. \quad \text{(B.10)}$$

Finally, obtaining $\langle N^2 \rangle$ from the expression of the standard deviation of the hypergeometric distribution:

$$\Delta^2 N = \langle N^2 \rangle - \langle N \rangle^2 = \frac{N_0 K}{M} \frac{(M-K)}{(M-1)} \left(1 - \frac{No}{M}\right). \quad \text{(B.11)}$$

Substituting it into eqn (B.10), we recover eqn (B.8), demonstrating the validity of the latter expression.

Three-Cell Interactions in T Cell-Mediated Suppression? A Mathematical Analysis of Its Quantitative Implications[1]

Kalet León,*† Rolando Peréz,† Agustin Lage,† and Jorge Carneiro[2]*

Aiming to further our understanding of T cell-mediated suppression, we investigate the plausibility of the hypothesis that regulatory T cells suppress other T cells (target cells), while both cells are conjugated with one APC. We use a mathematical model to analyze the proliferation inhibition scored during in vitro suppression assays. This model is a radical simplification of cell culture reality, assuming that thymidine incorporation is proportional to the number of target cells that would instantaneously form conjugates with APCs that are free of regulatory cells. According to this model the inhibition index should be mainly determined by the number of regulatory cells per APC and should be insensitive to the number of target cells. We reanalyzed several published data sets, confirming this expectation. Furthermore, we demonstrate that the instantaneous inhibition index has an absolute limit as a function of the number of regulatory cells per APC. By calculating this limit we find that the model can explain the data under two non-mutually exclusive conditions. First, only ~15% of APCs used in the suppression assays form conjugates with T cells. Second, the growth of the regulatory cell population depends on the target cells, such that the number of regulatory cells per APC increases when they are cocultured with target cells and overcomes its limit. However, if neither of these testable conditions is fulfilled, then one could conclude that suppression in vitro does not require the formation of multicellular conjugates. *The Journal of Immunology,* **2001, 166: 5356–5365.**

Several mechanisms contribute to natural tolerance to body tissues. Thymic deletion purges the emergent T cell repertoire from reactivities to peptides expressed intrathymically (1, 2). Mechanisms such as T cell ignorance (3), T cell anergy (4), and clonal exhaustion (5, 6) may contribute to the peripheral unresponsiveness to tissue-specific Ags that are not expressed in the thymus. However, these mechanisms do not seem sufficient to explain tolerance to continuous stimulation by self-Ags, as they can easily be overcome during the course of immune responses and inflammation (7). Dealing with this chronic stimulation by peripheral Ags seems to call into action yet another, maybe complementary, mechanism of T cell-mediated tolerance.

The involvement of T cells in natural tolerance was demonstrated by partial T cell depletion (8–10) or reconstitution of immunodeficient animals with fractionated peripheral T cell subpopulations (9, 11–15). These experiments demonstrated the existence in normal healthy animals of $CD4^+$ T cell subpopulations with the capacity to prevent autoimmune responses (12, 16) or uncontrolled inflammation triggered by other cells (7, 15). Despite their fundamental and clinical significance, such regulatory T cells have not yet been isolated or cloned, and their phenotype is still elusive.

Although the inability to isolate regulatory T cell has been an obstacle in the characterization of their mechanism of action, important clues have nevertheless been derived from recent in vivo and in vitro studies. In a system in which T cell-mediated tolerance is induced to skin grafts, Waldmann reported that suppression requires that the Ags recognized by tolerant and immune T cell populations are expressed on the same cells (17–19). This type of linked suppression was investigated by several in vitro systems, from systems using T cell lines (20, 21) to ex vivo systems where suppression in vivo and in vitro is well correlated (22, 23). All these studies, with the exception of a recent controversial result (24), indicate that suppression mediated by regulatory T cells in vitro requires direct cell-to-cell contact between APCs, regulatory T cells, and T cells that are targets of regulation (22, 23, 25). These observations led to the hypothesis that suppression takes place while the regulatory T cell and its target T cell are coconjugated with a single APC, i.e., suppression requires simultaneous interaction among three cell types in multicellular conjugates. Alternative hypotheses can be found in the literature, ranging from simple competition for conjugation with APC (20, 26) to more complex interactions in which the APC itself conveys a signal from the regulatory T cell to its target (27–30). The inability to experimentally assess the different hypotheses has prevented a definitive resolution of this controversy. They have been tested using complex experimental designs whose qualitative results do not have a single straightforward interpretation.

A way of overcoming this impasse is to test directly the hypotheses by their intrinsic capacity to predict and explain the quantitative details of the in vitro suppression assays. Demonstration that one or more hypotheses can be rejected due to incompatibility with experimental data may contribute to reduce the list of candidates. In this context, suppression dependent on simultaneous conjugation of regulatory and target T cells with the APC is an obvious testable candidate due to inherent probabilistic constraints. Mechanisms of interaction involving the formation of conjugates of more than two cell types have been postulated previously (31), the major example being the classical view of linked recognition of Ag

*Instituto Gulbenkian de Ciência, Oeiras, Portugal; and †Centro de Inmunologia Molecular, Habana, Cuba

Received for publication December 6, 2000. Accepted for publication February 16, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Program Praxis XXI of the Ministério para Ciência e Tecnologia, Portugal (Grant Praxis/P/BIA/10094/1998). J.C. was supported by Fundação para a Ciência e Tecnologia, Program Praxis XXI (Fellowship BPD/11789/97). K.L. was supported in part by the Fundação Calouste Gulbenkian.

[2] Address correspondence and reprint requests to Dr. Jorge Carneiro, Estudos Avançados, Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal. E-mail address: jcarneir@igc.gulbenkian.pt

aby CD4 and CD8 T cells and their cooperation. It has been argued (32, 33) that such interaction imposes strong constraints on the efficiency of the process where it is imbedded due to the relatively low frequency of its rate-limiting event, the simultaneous encounter between more than two cells. This straightforward quantitative prediction provides an easy way to test the involvement of multicellular conjugates in suppression.

We report here a mathematical analysis of the hypothesis that in vitro suppression takes place in multicellular conjugates and therefore is limited by their formation. We use a general model (34) describing the distribution of multicellular conjugates in a population of regulatory T cells, target T cells, and APCs. We first identify the generic quantitative predictions of the requirement for multicellular conjugates, and then test these predictions on several results of in vitro suppression assays reported in the literature.

Materials and Methods

In vitro suppression assay and inhibition index I

We model the suppression of the response of target cells by regulatory cells in vitro. This is quantified by the following assay. T_0 target cells are stimulated with A_0 APCs in the presence or the absence of R_0 regulatory cells in two parallel cultures that we call here, respectively, test and reference cultures. After a given amplification period, denoted Δ, the cultures are pulsed with [^{3}H]thymidine for a short period, τ, to assess the number of dividing cells. The amount of [^{3}H]thymidine incorporated in the cell fraction during the pulse is quantified. Typically a quantity that we call here the experimental inhibition index, I, is obtained by dividing the counts in the test culture by the counts in the reference culture: I = cpm[test culture]/cpm[reference culture] (Equation 1). Note that this index is inversely related to the actual suppression or inhibition (which can be estimated as 1 − I).

The results of in vitro suppression assays are typically presented as plots of the experimental inhibition index vs the ratio between the numbers of regulatory and target cells originally put into culture R_0/T_0.

The data sets used in this article are listed in Table I and were obtained by the following experimental systems.

System A. The experiments were reported by Lechler and colleagues (20). Briefly, a standard in vitro suppressor assay was performed using the T cell line HC3 as a source of target T cells. The regulatory cells are HC3 cells previously rendered anergic in vitro. The APCs were irradiated B-LCLs cells pulsed with a peptide agonistic for HC3 cells. The cultures were pulsed between 48–66 h of culture. Different clone-specific T cells lines were used (including some alloreactive ones) with equivalent results. The major drawback of this experimental system for understanding T cell-mediated suppression is that there is not enough evidence that anergic T cells act as regulatory cells in vivo.

System B. The experiments were reported by Powrie and colleagues (25). CD4 T cells from a normal healthy mouse were sorted into CD45RB^{low} CD38$^+$ and the remaining subpopulation. The first subpopulation was used as regulatory cells, and the second subpopulation was used as target cells in the suppression assay. The cells were stimulated with anti-CD3 using irradiated T lymphocyte-depleted spleen cells as APCs. The cultures were pulsed between 60–78 h of culture. The main drawbacks of these experiments are that there is no clear relationship between the inhibition index in vitro and the immunosuppression in vivo, and the population of CD4$^+$CD45RB^{low}CD38$^+$ T cells is enriched, but not pure, in regulatory T cells.

System C. The experiments were reported by Sakaguchi et al. (23) and Thornton and Shevach (22). CD4$^+$ CD25$^+$ T cells were used as regulatory cells, and CD4$^+$CD25$^-$ T cells were used as target cells in the suppression assay. The stimulus was again soluble anti-CD3 and irradiated T cell-depleted spleen cells. The thymidine pulse was given between 66–72 h of culture. The major advantage of this system is that that there is a good correlation between the results of the in vitro suppression assay and the results of adoptive transfers in vivo. Its main drawback is that neither the regulatory nor the target T cell population can be assumed to be pure. Indeed, both authors stressed the fact that the CD4$^+$CD25$^+$ population is enriched in regulatory cells, but is not pure. The actual purity of regulatory cells is unknown.

A mathematical model of in vitro proliferative responses dependent on the formation of multicellular conjugates

Modeling the in vitro suppression assay and the inhibition index requires a quantitative description of the proliferative response in the individual reference and test cultures. In this section we describe a model of cell proliferation in a culture containing APCs, regulatory cells, and target cells. In the next section we use this model to derive a quantity corresponding to the inhibition index.

The elementary processes and interactions underlying this model are illustrated in Fig. 1. The main postulate, Postulate 1, is that T cell proliferation requires productive conjugation with the APC, and interactions between regulatory and target cells require that both cells are simultaneously conjugated with the APC, i.e., the formation of multicellular conjugates. Following this postulate the number of cells proliferating in a culture that contains, at a given time, A APCs, R regulatory cells, and T target cells is given by

$$P(A, T, R) = \sum_{i=0}^{s} \sum_{j=0}^{s-i} \alpha_{i,j} \cdot A_{i,j}(A, T, R) \qquad (2)$$

where $A_{i,j}(A,T,R)$ is the number of conjugates containing one APC, i target cells, and j regulatory cells in the culture, $\alpha_{i,j}$ are proliferation coefficients determined by the stoichiometry of each conjugate, and s is the number of conjugation sites per APC. Each coefficient $\alpha_{i,j}$ counts how much proliferation is obtained from a conjugate containing i target cells and j regulatory cells. From Equation 2 it is easy to understand that we need to specify the values for every $A_{i,j}(A,T,R)$ and the proliferation coefficients $\alpha_{i,j}$.

The conjugates with a given stoichiometry present in a culture can be counted using a formalism we proposed previously (34). This formalism is valid as long as the cell cultures fulfill the following additional postulates.

Postulate 2. Following previous suggestions (35, 36), we assume that the s conjugation sites on each APC are independent and equivalent with either regulatory or target T cells according to conjugation constants K_R and K_T, respectively. Postulate 3, the conjugation constants are formally analogous to molecular affinities and describe the equilibrium numbers of conjugated target and regulatory T cells (respectively, T_c and R_c), the numbers of free target and regulatory T cells (respectively, T-T_c and R-R_c), and the number of total free conjugation sites ($s.A$-Rc-Tc). Thus, they are defined as:

$$K_T = \frac{T_c}{(T - T_c)(sA - R_c - T_c)} \qquad (2a)$$

$$K_R = \frac{R_c}{(R - R_c)(sA - R_c - T_c)} \qquad (2b)$$

Postulate 4. The processes of formation and dissociation of conjugates are very fast processes compared with activation, proliferation, differentiation, and death of T cells. To a good approximation a mixture of cells at any given time will fulfill the equilibrium conditions of Equation 2.

Following these assumptions we calculate the conjugates in two steps.

Table I. *Experimental data sets*

System	A_0	T_0/A_0	Inhibition index at the indicated value of the ratio R_0/T_0					
			R_0/T_0 = 0.06	R_0/T_0 = 0.12	R_0/T_0 = 0.25	R_0/T_0 = 0.5	R_0/T_0 = 1	R_0/T_0 = 3
A	30,000	0.16					0.35	0.05
B	1,000,000	0.1		0.75	0.47	0.26	0.19	
C-1	50,000	0.5		0.42	0.22	0.14	0.02	
C-2	50,000	1.0	0.35	0.23	0.12	0.023		

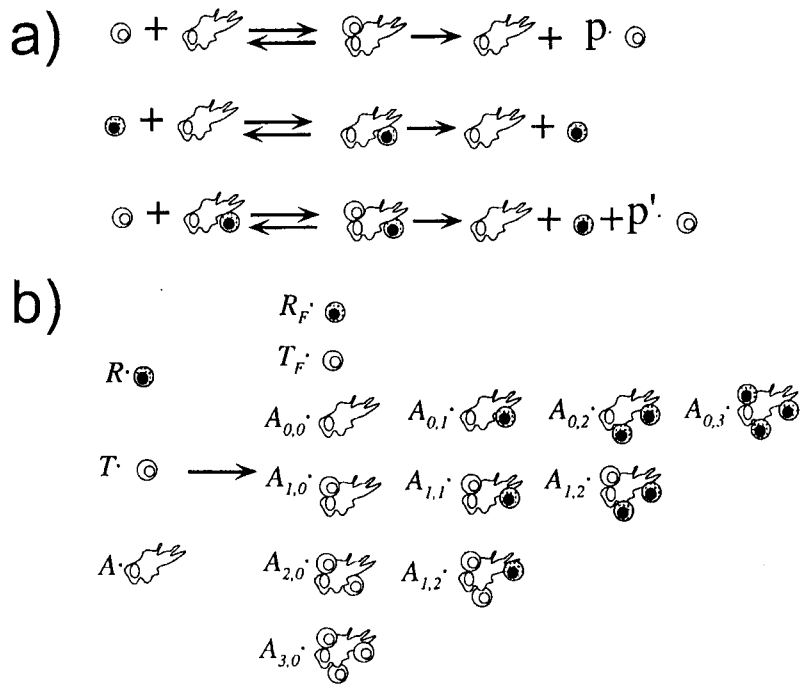

FIGURE 1. Reaction diagrams illustrating the elementary processes and interactions in the model. *A*, A target cell (white) conjugates with an APC and dissociates at a fast rate; following productive conjugation with the APC each target cell will give raise to a progeny *p*. Regulatory cells (gray) conjugate with the APC and dissociate at a fast rate; following conjugation with the APC regulatory cells will not proliferate. If a target cell conjugates with an APC conjugated with at least one regulatory cell, the target cell will give rise to a lower progeny *p'*, due to inhibitory interactions. *B*, In a mixture containing total *A* APCs, *R* regulatory cells, and *T* target cells a rapid quasi-steady composition will be reached containing nonconjugated regulatory cells, target cells, and APCs (respectively, R_F, T_F, and $A_{0,0}$) and several multicellular conjugates $A_{i,j}$ containing one APC, *i* target cells, and *j* regulatory cells.

First, the numbers of conjugated target and regulatory cells, T_c and R_c, respectively are calculated as a function of *A*, *R*, and *T* by solving Equation 2. A general solution for T_c and R_c was previously presented (34). Throughout this article we will consider two particular cases: one in which the conjugation constants of regulatory and target cells are identical, $K_R = K_T$ (Equation 3), and one in which the conjugation constant of regulatory cells is infinite, $K_R = \infty$ (Equation 4).

$$R_C = \frac{R}{R+T}\cdot \frac{K_R(sA+T+R)+1-\sqrt{(K_R(sA+T+R)+1)^2-4K_T^2sA(T+R)}}{2K_R},$$

$$T_C = \frac{T}{R}R_C \tag{3}$$

$R_C = \mathrm{MIN}(R, sA)$,

$$T_C = \frac{K_T(sA-R+T)+1-\sqrt{(K_T(sA-R_C+T)+1)^2-4K_T^2(sA-R_C)T}}{2K_T} \tag{4}$$

Considering these two cases is appropriate because, intuitively, increasing the conjugation constant of regulatory cells will increase the instantaneous inhibition. Setting K_R at infinity is certainly unrealistic, because it means that regulatory cells would be permanently "glued" to the APC and could not be displaced by target cells. However, a consideration of both cases helps us to keep track of the more realistic situation that can be expected to lie in between these two extremes.

Once we obtain the total number of conjugated target and regulatory cells we calculate how these are distributed among the *A* sets of *s* equivalent sites. The result is a product of two hypergeometric distributions:

$$A_{i,j}(A,T,R) = H[i+j, T_c+R_c, sA, s]\cdot H[i, T_C, T_c+R_c, i+j] \tag{5}$$

where H is defined as

$$H[l,k,m,n] = \binom{k}{l}\binom{m-k}{n-l}\Big/\binom{m}{n} \tag{6}$$

We turn now to the problem of specifying the proliferation coefficients $\alpha_{i,j}$. This requires three additional postulates about the actual interactions taking place in the conjugates.

Postulate 5. The probability that a target cell proliferates following a productive conjugation with an APC is independent of the number of targets cells *i* in the same conjugate. In other words, there is no cooperativity among target cells.

Postulate 6. The regulatory cells do not proliferate following conjugation with the APC.

Postulate 7. If a target cell participates in a conjugate that contains at least one regulatory cell, then its conjugation is not productive, i.e., a single regulatory cell is able to prevent the proliferation of all the target cells in the same conjugate.

Following these postulates we specify the coefficients as follows:

$$\alpha_{i,j} = \begin{cases} i\cdot p & \text{if } j=0 \\ 0 & \text{if } j\geq 1 \end{cases} \tag{7}$$

where *p* is the proliferation per productively conjugated target cell.

Postulates 5–7 allow a major simplification of the intercellular interactions. Postulate 6 is based on the fact that regulatory cell populations do not proliferate when cultured with APCs in the absence of target cells (16, 20, 23, 25). Postulate 7 maximizes the strength/efficiency of the inhibition per regulatory cell, and this is adequate for our purpose of identifying a limit in the efficiency of suppression dependent on the formation of multicellular conjugates.

Instantaneous inhibition of proliferation and its index λ(A,T,R)

To compare the predictions of the models with the experimental inhibition index we devised a theoretical quantity that we call instantaneous inhibition index $\lambda(A,T,R)$, defined as the ratio between the proliferation in an ideal test culture containing at a given time *A* APCs, *T* target cells, and *R* regulatory cells and the proliferation in an ideal reference culture that contains at the same time only *A* APCs and *T* target cells:

$$\lambda(A,T,R) = \frac{P(A,T,R)}{P(A,T,0)} = \sum_{i=1}^{s} i\cdot A_{i,0}(A,T,R)\Big/\sum_{i=1}^{s} i\cdot A_{i,0}(A,T,0) \tag{8}$$

The instantaneous inhibition index estimates the fraction of target cells that is not prevented from having productive conjugation with the APC by the presence of a given number of regulatory cells in the test culture compared with the reference culture. Substitution of Equation 5 in Equation 8 and rearrangement simplify to the product of two factors:

$$\lambda(A,T,R) = \theta\cdot\chi \tag{9}$$

The factor χ is the ratio between the numbers of target cells conjugated in the ideal test and reference cultures:

$$\chi(A, T, R) = \frac{T_C(A, T, R)}{T_C(A, T, 0)} \quad (10)$$

where $T_C(A,T,R)$ is generically the number of conjugated target cells in a mixture containing A, R, and T cells calculated according to Equation 3 or 4.

The factor θ is simply the fraction of target cells participating in conjugates free from regulatory cells in the ideal test culture:

$$\theta = H(0, R_C, sA, s)\frac{sA}{sA - R_C} \quad (11)$$

The factors χ and θ represent, respectively, the suppression by competition for conjugation sites and the suppression by active inhibition in multicellular conjugates.

The instantaneous inhibition index can be calculated at any given time for ideal reference and test cultures that have, by construction, the same numbers of target cells and APCs. The comparison of this quantity to the real experimental inhibition index is not straightforward. Although equal numbers of APCs and target cells are put into the reference and test cultures, at the time of the pulse (i.e., after the amplification period) these numbers may have changed due to the interactions and processes, such as proliferation and death in the culture. Keeping this problem in mind, we will equate the inhibition index to the instantaneous inhibition index estimated from the numbers of APCs, target cells, and regulatory cells put into culture, respectively, A_0, T_0, and R_0.

$$I \equiv \lambda(A_0, T_0, R_0) \quad (12)$$

This approximation implies two additional postulates.

Postulate 8. The duration of the pulse of thymidine incorporation τ is negligible compared with cell population doubling time.

Postulate 9. The numbers of APCs, target cells and regulatory cells do no change during the amplification period Δ. As a corollary, the duration of the amplification period Δ is negligible.

Note that although this approximation is a strong mathematical simplification, it may nevertheless be biologically reasonable. Thus, it is commonly assumed that following stimulation by an APC a T cell may undergo several rounds of division without further interactions between its progeny and the APC (this assumption is substantiated by Ref. 37). Under these conditions only the initial interaction of the T cells with the APC would be relevant.

More rigorous analysis of the results could be made using ordinary differential equations to follow the actual processes of growth and death of the T cell populations during the amplification period Δ and the total number of cells incorporating thymidine in the period τ. The approximation used here, similar to that followed by Borghans et al. (38), consists of calculating the ratio between the positive component of the derivative of the target cells in the test and reference cultures at the limit when both Δ and τ tend to zero (Postulates 8 and 9). There is more to be said about the accuracy of this approximation that will be taken up in *Discussion*.

Model solving and fitting to experimental data

All the mathematical analyses, both analytic and numeric, were performed with the software Mathematica 3.0 from Wolfram Research (Champaign, IL). The comparison of the models with the experimental data was performed assuming that the populations that were used as sources of APCs, regulatory cells, or target cells in Table I were 100% pure.

Results

Competitive and active inhibitions contribute to suppression, but one of these components may predominate

According to our model a regulatory cell prevents, by a direct active inhibitory interaction, the proliferation of all target cells that participate in the same multicellular conjugate with the APC. Because regulatory and target cells share the conjugation sites on the APC, a regulatory cell will also inhibit its targets, in an indirect way, when it occupies a site on the APC. These two modes of inhibition, active and competitive, are inevitably associated, because for a regulatory cell to inhibit actively its targets it also needs to occupy a site. Understanding how much of the suppression can be explained by competition alone and how much can be explained by active inhibition becomes a fundamental issue at this stage. This issue is particularly relevant if we consider that competitive inhibition and active inhibition in multicellular conjugates are discussed in the literature as explanations for linked suppression (20, 39).

The first result of our quantitative analysis is the actual derivation of an expression of the instantaneous inhibition index, λ, as the product of two factors, θ and χ, representing, respectively, the contributions of active and competitive inhibition (Equation 9). The presence of the number of conjugated regulatory cells, R_c, in the expressions for both θ and χ reveals the interdependence of the two components of suppression (Equations 10 and 11). Both factors vary between 0 and 1; therefore, as expected, suppression including active inhibition is always more efficient than one based on competitive inhibition alone.

The second result of our analysis is that although both competitive and active inhibitions can potentially contribute to in vitro suppression of target cell by regulatory cells, in practice one of these components may predominate depending on the composition of the culture. The interplay between these two components of suppression is illustrated in Fig. 2 (*top*) where we represent the total suppression, $\theta \times \chi$, and the suppression due to competitive inhibition alone, χ. The contribution of competitive inhibition becomes significant only when the number of free sites on the APCs becomes reduced, i.e., at a relatively high number of total T cells (regulatory and target) compared with the total number of conjugation sites ($s \times A$). However, active inhibition is already significant when the number of conjugated regulatory cells is on the same order as the number of APCs. Changing the parameters in the system modulates the interplay between the two components. For example, the higher the number of sites per APC, the higher the

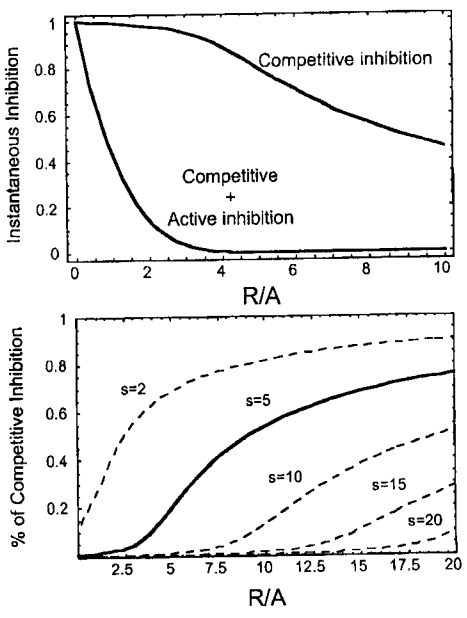

FIGURE 2. Competitive and active inhibitions are potential components of suppression. *Top*, Competitive inhibition, χ (dashed line), and total instantaneous inhibition, λ ($=\theta \times \chi$, solid line), are plotted as a function of the number of regulatory cells per APC (R/A). Parameters: $K_E = K_R = 1$, $s = 5$, $A = 10$, $T = 10$. *Bottom*, The percent suppression due to competitive inhibition (calculated as $(1 - \chi)/(1 - \theta \times \chi)$) is plotted as a function of the indicate values of the ratio R/A. The solid line corresponds to the same parameter settings as graph A, whereas the dashed lines vary the numbers of sites per APC, s, as indicated.

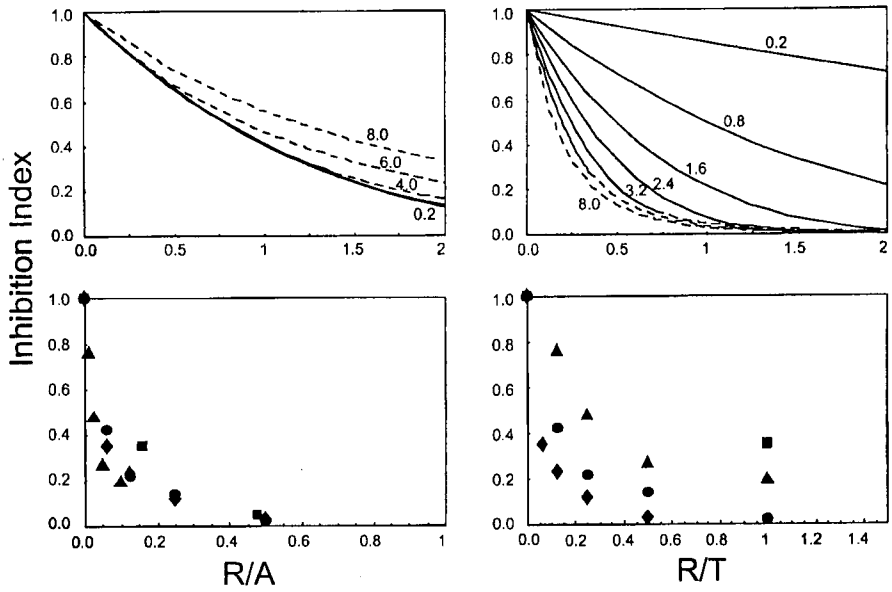

FIGURE 3. Competitive and active inhibitions have different sensitivities to changes in the number of target cells in the culture. *Top panel*, The instantaneous inhibition index, λ, is plotted as a function of the ratio *R/A* (*left*) or as a function of the ratio *R/T* (*right*) for the indicated values of the number of target cells per APC (*T/A*), and for $s = 5$ conjugation sites per APC. Solid and dashed lines indicate that the percentage of suppression due to competition is, respectively, lower and higher than 10%. The model predicts that in the representation on the *left*, the curves overlap while competition is negligible, i.e., when active inhibition predominates. In contrast, in the representation on the *right*, the curves overlap when competition is significant, i.e., when direct inhibition is negligible. Basic parameters are as given in Fig. 2*A*. *Bottom panel*, The experimental inhibition index, *I*, is plotted as a function of the ratio *R/A* (*left*) or *R/T* (*right*) in the culture for the data sets listed in Table I: *A*, squares; *B*, triangles; *C1*, diamonds; and *C2*, circles. The curves lie closer in the *R/A* representation than in the *R/T* representation, suggesting a predominance of active inhibition over competitive inhibition.

proportion of suppression due to active inhibition and the lower the proportion due to competitive inhibition (Fig. 2, *bottom*).

Competitive inhibition and active inhibition have different sensitivities to changes in the numbers of target and regulatory cells

Having demonstrated that suppression may be explained by competitive and/or active inhibitions and that either of these components may predominate depending on the culture conditions, we tried to devise a strategy to clarify under which regimen the in vitro culture system is operating.

To do this we consider the properties of two ideal extreme cases in which suppression is exclusively explained by either competitive inhibition or active inhibition. For an ideal case of suppression by competitive inhibition alone, we set θ to unity and assumed additionally that target cells in both test and reference cultures were in large excess compared with a limited number of conjugation sites, such that the number of free conjugation sites was negligible. Under such conditions, the expression for instantaneous suppression, λ, simplifies to:

$$\lambda = 1 \cdot \chi \approx \frac{1}{1 + \frac{K_R}{K_T} \cdot \frac{R}{T}} \qquad (13)$$

This expression is dependent on the ratio between the numbers of regulatory and target cells weighted by their respective conjugation constants. This dependence on both cell populations reflects the intrinsic symmetry in competitive interactions; regulatory cells may compete out their targets, but they can also be competed out. Competitive inhibition is expected to be comparably sensitive to changes in the numbers of regulatory and target cells as long as the conjugation constants are not very different. If suppression is dominated by competitive inhibition, then the inhibition index should have a similar sensitivity pattern, which would be adequately captured by plotting the index as a function of the ratio *R/T*.

In the other case in which suppression is explained exclusively by active inhibition the expression for λ reduces to:

$$\lambda = \theta \cdot 1 = H(0, R_C, sA, s)\frac{s}{s - \frac{R_C}{A}} \qquad (14)$$

This expression is, by construction, independent of the number of target cells. Hence, in the absence of competition between regulatory and target cells for conjugation sites, there is no way in which target cells can interfere with regulatory cells. Under these conditions the number of conjugated regulatory cells, R_c, is determined only by the total number of regulatory cells, *R*, the number of sites, $s \times A$, and the conjugation constant, K_R. Furthermore, a simple numerical analysis shows that expression 14 is just a function of the ratio *R/A* when the number of APCs is large enough ($A > 100$), and R_c is proportional to *R* (which is always true in the absence of competition, i.e., $R_c \ll s \times A$). The insensitivity of active inhibition to changes in the number of target cells is qualitatively easy to understand if we consider that it is the result of an intrinsically asymmetric interaction by which regulatory cells inhibit, but are not affected by, their targets. Therefore, when active inhibition predominates, suppression is expected to be a simple function of the number of regulatory cells per APC (*R/A*) and to be insensitive to changes in the number of target cells.

These predictions are illustrated in the theoretical curves of Fig. 3 (*top panel*) using the general expression for the instantaneous inhibition, λ (Equation 9). λ is plotted as a function of the ratio of regulatory cells per APC (*R/A*) or as a function of the ratio regulatory cells per target cells (*R/T*) for several numbers of target cells. In the *R/A* representation the different curves overlap when

active inhibition predominates (lines are continuous) and start diverging when competition becomes effective (lines are dashed). In contrast, in the *R/T* representation the curves diverge when competition is negligible (continuous) and converge when competition is more significant (dashed). The dispersion of different experimental curves may therefore provide clues about the relative contributions of competitive and active inhibitions to suppression.

The inhibition index scored by in vitro suppression assays is relatively insensitive to changes in the number of target cells

Following the theoretical considerations of the previous section we asked which representation, *R/A* or *R/T*, reduces the dispersion of the experimental inhibition index. A plot of the experimental data sets (Table I) in the coordinates *I* vs *R/A* and in the *I* vs *R/T* shows that experimental points are significantly less dispersed in the first coordinate system compared with the second (Fig. 3, *bottom panel*). This is particularly meaningful considering that the data are obtained with different experimental systems and by different laboratories. Moreover, data points resulting from the same experimental system, such as C1 and C2 (circles and diamonds), which are separate in the *I* vs *R/T* coordinate system appear as if they belong to the same curve in the *R/A* representation.

This graphic analysis indicates that the experimental inhibition index is relatively insensitive to changes in the numbers of target cells. This sensitivity pattern suggests that suppression is based on an active inhibitory effect of regulatory cells on target cells, and that competition for conjugation sites plays a minor role. Alternatively, the insensitivity to the number of target cells may result from competition under the particular condition in which the conjugation constant of the regulatory cells is much higher than the conjugation constant of target cells.

The results of the suppression assay are traditionally represented in the coordinates *I* vs *R/T* (20, 23, 25), a procedure that stems historically from the cytotoxic assay. Our results indicate that the coordinates *I* vs *R/A* are a better representation of the experimental data than the classical coordinates *I* vs *R/T*, because the second representation introduces an artificial effect of the number of target cells. Heretofore we adopt the first representation. In the next section we demonstrate that the instantaneous suppression has an absolute limit that is readily revealed in these coordinates.

The instantaneous inhibition index has an absolute asymptotic limit as a function of the number of regulatory cells per APC

As stated in the introduction, the efficiency of suppression by active inhibition should be limited by the frequency of multicellular conjugates. In this section we show that instantaneous inhibition has such a limit, and we estimate its value as a function of the parameters involved. According to the model the instantaneous inhibition, λ, depends on three parameters: the number of conjugation sites per APC, s, and the conjugation constants K_R and K_T for regulatory and target cells, respectively. These three parameters are explored aiming to identify those values that minimize λ.

Two prototypic combinations of the values of K_R and K_T are considered: equal conjugation constants of regulatory and target cells and an infinite conjugation constant for regulatory cells. A consideration of the two cases allows us to keep track of the more realistic situation that can be expected to exist in between these two extremes. The conclusions drawn from these particular cases in this section are general.

The parameter dependence of instantaneous inhibition, λ, is illustrated for these two cases in Fig. 4. In the *top panel*, the ratio *R/A* required to reach 50% inhibition ($\lambda = 0.5$) is plotted as a function of the ratio *T/A* for different values of K_T. In the case of equal conjugation constants (Fig. 4, *left*), the bigger the value of K_T, the smaller the ratio *R/A* required to reach 50% inhibition. Moreover, as K_T increases, the curves converge to a limit curve

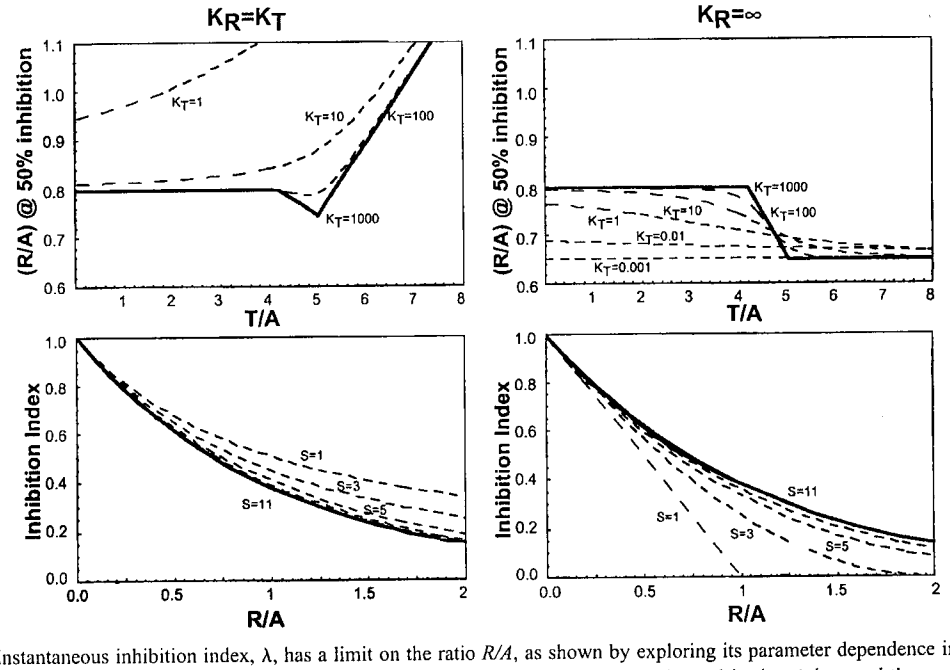

FIGURE 4. The instantaneous inhibition index, λ, has a limit on the ratio *R/A*, as shown by exploring its parameter dependence in two extreme cases. In the *left panel* the conjugation constants of regulatory and target cells are identical ($K_T/K_R = 1$), and in the *right panel* the conjugation constant of regulatory cells is infinite ($K_T/K_R = 0$). *Top*, The ratio *R/A* required to obtain 50% inhibition ($\lambda = 0.5$) is plotted as a function of *T/A* for the indicated values of the conjugation constant of target cells (K_T) and for a particular number of conjugation sites per APC, $s = 5$. A minimal value of *R/A* is required to reach 50% for any possible values of K_T and *R/T* indicated by an arrow. *Bottom*, Limit suppression curves are depicted for the indicated values of the conjugation sites per APC, s. These curves are the percentage of instantaneous inhibition plotted as a function of the minimal value *R/A* that is required to reach this percentage regardless of the values of K_T and *T/A*. (As in Fig. 3 solid and dashed lines indicate that the percent suppression due to competition is, respectively, lower and higher than 10%.)

corresponding to the situation when K_T = infinity. This limit curve defines the minimal value of the ratio *R/A* that can lead to 50% inhibition for a particular number of conjugation sites per APC, *s*. The absolute minimum is obtained at $T/A = s$. The shape of the limit curve gives some information about the processes taking place in the system. For low values of the ratio *T/A*, the curve is a horizontal line. The number of T cells is so small that there is no competition for the conjugation sites on the APC. To reach 50% inhibition one would need to have enough regulatory cells so that about 50% of APC are conjugated with at least one regulatory cell and about 50% are free from regulatory cells. For a short interval of intermediate *T/A* values there is a transition phase that takes place while the conjugation sites per APC get saturated. During this phase, increases in the number of target cells means that they are occupying the free sites on an APC already containing regulatory cells; this maximizes the inhibitory effect per regulatory cell. For high values of the ratio *T/A* the curve becomes linear in a regimen in which competition predominates in the system. Any increase in the number of target cells displaces regulatory cells from the APC. To keep the level of inhibition at 50%, the number of regulatory cells must be increased proportionally to the target cells to keep their balance in conjugates.

In the case of infinite conjugation constant of regulatory cells (Fig. 4, *top right*) the picture is different. In contrast with the previous case, for low values of *T/A*, the higher the value of K_T, the lower the value *R/A* required to obtain 50% inhibition. For intermediate values of the ratio *T/A*, there is a value of K_T that gives the minimum ratio of *R/A*. For very high values of *T/A* the decrease in K_T entails an increase in the ratio *R/A* required to reach 50% suppression. As K_T tends to infinity, a limit curve is obtained. The shape of this limit curve resembles that obtained with equal conjugations constants, but here, because target cells cannot by definition compete out the regulatory cells, the linear phase corresponding to competition has a slope of zero. From examination of Fig. 4 (*top right*) it is clear that the absolute minimum *R/A* required for 50% suppression is obtained when K_T = infinity, and *T/A* is greater than *s*. The same absolute minimum is also obtained when K_T tends to zero, and a limit horizontal line is obtained, although this limit $K_T = 0$ is biologically uninteresting because it would imply no proliferation in the cultures.

The consideration of these prototypical cases reveals the existence of an absolute minimal number of regulatory cells per APC that can lead to 50% inhibition. In the case where APCs have five conjugation sites, 50% suppression can only be scored only if the ratio *R/A* is greater than 0.73 in the case of $K_R = K_T$ and 0.65 in the case of K_R = infinity. Although these absolute minima were illustrated in Fig. 4*A* for a particular number of conjugation sites per APC, *s*, and a given inhibition index, λ, their existence is generic. For any given value of *s*, any chosen value of λ cannot be obtained if the ratio *R/A* is lower than a corresponding minimum ratio *R/A*. Therefore, we can define for each value *s* a corresponding limit curve relating the minimal *R/A* ratio required for scoring a given instantaneous inhibition index. In Fig. 4 (*bottom*) these limit curves are plotted for a series of values of *s* for the two cases under study. In the case of equal conjugation constants (Fig. 4, *bottom left*) as *s* increases the curves move down and tend asymptotically to an absolute limit curve that is reached when s = infinity. In the case of infinite conjugation constant (*bottom right*) the curves move up as *s* increases, reaching the same limit when s = infinity.

In summary, the results in this section show that there is an asymptotic lower limit to the instantaneous suppression λ as a function of the ratio *R/A* in the test culture. In other words, independently of parameter settings, any chosen value of λ requires a minimum ratio *R/A*. In the following section this limit of the instantaneous inhibition index is compared with the experimental data.

The instantaneous inhibition model fails to fit the experimental inhibition index

In this section we ask how the experimental data relate to theoretical limit curve identified in the previous section. To do this we will equate the experimental inhibition index I to the instantaneous inhibition index, λ, according to Equation 12. This is a gross approximation that requires Postulates 8 and 9, which assume that the duration of the period before the thymidine pulse and that of the actual pulse are negligibly. Additionally, we assume that the populations of regulatory cells, target cells, and APCs are 100% pure. Under these assumptions, in Fig. 5 (*top*) the markers represent the experimental data for the inhibition index I during in vitro suppression assays reported by several groups (Table I), and the lines represent the theoretical limit curves for equal conjugation constants and for infinite conjugation of regulatory cells (i.e., the *leftmost* curves in the *bottom panel* of Fig. 4). All the experimental points lay outside the range defined by the limit curve of the instantaneous inhibition index predicted by the model. Therefore, the conclusion is that the instantaneous inhibition index cannot be fitted to the available experimental data. It is worth estimating the gap between the experimental data and the limit curve. This can be quantified by determining a multiplication factor to apply to the R_0/A_0 ratio allowing a corrected experimental curve to overlay the theoretical limit curves. This factor f is ~6 in the case of an infinite conjugation constant for regulatory cells (Fig. 5, *middle*) and ~10

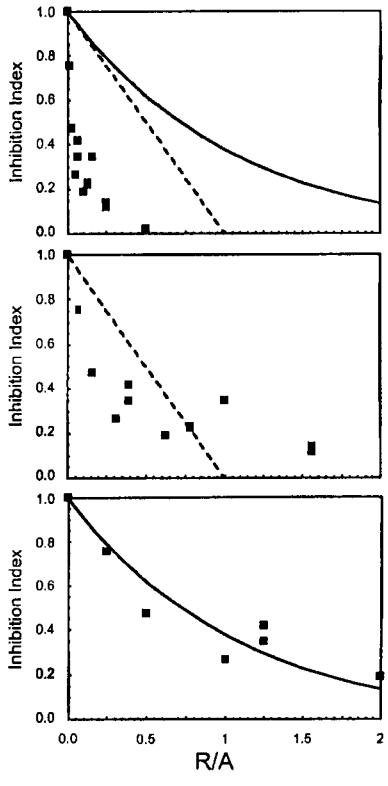

FIGURE 5. Comparison of the instantaneous inhibition index, λ, and the experimental inhibition index, *I*. The lines represent the limit curves of λ as a function of *R/A* in the two extreme cases $K_T/K_R = 1$ (solid) and $K_T/K_R = 0$ (dashed). The squares represent the experimental data sets from Table I, assuming that the ratio of regulatory cells per APC in the cultures needs to be corrected by a factor such that $R/A = f \times R_0/A_0$, with $f = 1$ (*top*; same as in Fig. 3), $f = 6$ (*middle*), and $f = 10$ (*bottom*).

in the case of equal conjugation constants of regulatory and targets cells (Fig. 5, *bottom*).

There are three non-mutually exclusive interpretations for the failure to explain the experimental data. The numbers of cells may have been incorrectly estimated in the experimental assay, such that the true ratio R/A was, in fact, $f \times R_0/A_0$. Alternatively, some of the approximations in the model or in the procedure for mapping its predictions to the experimental data may be unrealistic. During the period before the thymidine pulse the ratio R/A may have changed by a factor f, violating Postulate 9. Finally, suppression in vitro may proceed by a mechanism that does not require formation of multicellular conjugates.

Discussion

With the purpose of better understanding linked suppression, we addressed the quantitative predictions of the hypothesis that regulatory cells suppress other T cells while both cells are conjugated with the APC. We analyzed the experimental inhibition index in cocultures of regulatory cells, target cells, and APCs using a simple mathematical model. This model is a radical simplification of the reality of cell cultures, as it assumes that thymidine incorporation is proportional to the number of target cells that would instantaneously form conjugates with APCs that are not simultaneously conjugated with regulatory cells. This simplification proved useful, because it allowed us to predict and to explain an insensitivity of the experimental inhibition index to changes in target cell number. However, it is bluntly unable to explain the actual percentage of suppression observed experimentally.

Our theoretical analysis has shown that both competition and active inhibition will quantitatively contribute to the final suppression. To identify whether one or the other component predominates, we put forward a method based on the sensitivity of inhibition index to changes in the number of regulatory cells per APC and in the number of regulatory cells per target. This strategy is only valid when the conjugation constants of the regulatory and target cells are on the same order of magnitude. Theoretically when the conjugation constant of regulatory cells is several orders of magnitude greater than that of target cells our criterion to distinguish the two components is not effective because competition itself becomes insensitive to changes in the number of target cells. How plausible is this extreme case? The conjugation between cells is determined by coordinated signaling and expression of multiple molecules in both cells (40, 41) Conjugation constants are mapped to the average duration of conjugates between two cells; the longer the conjugation constant, the longer the duration of the conjugate. Orders of magnitude differences in conjugation constants require orders of magnitude differences in conjugation times. Conjugation times of T cells with their APC were estimated to range from a few hours to less than a day (42, 43). Therefore, it difficult to squeeze within this interval two values of conjugation constants whose ratio is practically zero. Moreover, in some of the data sets used here regulatory cells exhibit an activated/memory phenotype ($CD45RB^{low}$ or $CD25^{+}$), while their targets are essentially naive cells ($CD45RB^{high}$ or $CD25^{-}$). Following these considerations it is unlikely that the conjugation constants of regulatory and target cells differ by orders of magnitude. Full activation of naive cells requires longer conjugation time with the APC than activation of blast or memory cells (42). Thus, eventual differences between conjugation constants of regulatory and target cells may actually be the opposite of that required for competition to be insensitive to changes in target cell numbers. Therefore, in the context of our model, this insensitivity must be interpreted as a predominance of an active inhibitory signal of regulatory delivered to target cells in multicellular conjugates.

Hitherto, it had gone unnoticed in the literature that the inhibition scored in suppression assays is mainly dependent on the number of regulatory per APC and is insensitive to changes in the number of target cells. This is an important finding by itself regardless of the particular explanation we proposed here. Any candidate mechanism for the interactions between regulatory and target cells must be able to account for this property. Generically, any unidirectional interaction (i.e., from regulatory to target cells) whose rate-limiting step is mediated by the APC can potentially explain insensitivity to the number of target cells. This may be true whether the APC mediates the interaction by bringing the two cells together (as studied here) or by conveying a signal from one cell to the other. In contrast, a mechanism involving symmetric interactions between regulatory and target cells, such a competition, can be expected to have difficulty explaining different sensitivities to the numbers of two cell types. We analyzed here competition for conjugation sites, but similar results can be expected for symmetric competition for survival or growth factors (20). Also, interaction mechanisms in which the rate-limiting step is a direct encounter between regulatory and target cells to form 1:1 conjugates (24) may lead to comparable sensitivities to the two cell populations and therefore be incompatible with the result reported here. Due to its relevance for hypothesis testing the relative sensitivities of in vitro inhibition to the three experimental variables (APCs, target and regulatory cell numbers) deserves more experimental attention.

We now turn to the failure to explain the experimental data by instantaneous inhibition. As stated in *Results*, this can be interpreted in at least three non-mutually exclusive ways. Each of these possibilities is plausible, nontrivial, and biologically relevant, and therefore deserves detailed discussion. However, before such discussion a methodological point deserves being stressed. We used a model that was radically simple both in structure and in the way it was compared with the experimental data. Nevertheless, in the context of this model the actual failure to explain the data becomes informative, because it suggests straightforward extensions that represent quantitative testable hypothesis about the mechanism of suppression. This illustrates a noteworthy aspect of mechanistic modeling of data: even negative results are often informative.

In all the experimental results listed in Table I it is quite plausible that there was an overestimation of the numbers of regulatory cells, target cells, or APCs that effectively participate in the suppression. Considering these possibilities it is quite straightforward to realize that assuming that the number of regulatory or target cells is overestimated (i.e., assuming that the number of T cells per APC is lower) does not help to bring the theoretical limit curve closer to the experimental data. Actually, in the case of regulatory cells it will just make things worst. In contrast, assuming that there is an overestimation in the number of APCs that form conjugates with T cells can reduce the gap between the prediction of the models and the experimental data. The correction factor estimated before implies that only ~15% of the APC used in suppression assays is not forming conjugates with T cells. This interpretation may be quite reasonable in the experimental systems where the APCs were obtained in vivo (e.g., T cell-depleted splenocytes) or where the APCs are a cell line loaded with an agonist peptide. In other systems in which the APCs are a cell line expressing a particular allogeneic MHC (21) it is more difficult to envisage that the population of APCs is not homogeneous in their capacity to form conjugates with T cells.

Equating the experimental inhibition index, I, to the instantaneous inhibition index calculated for the numbers of cells originally placed in culture is a radical simplification of reality. In all the systems studied here the suppression assay takes about 3 days

of culture. During this period it is possible that regulatory and/or target cells undergo several rounds of division, and/or that inhibitory effects are amplified before the thymidine pulse is performed. Regulatory cells might alter the growth kinetics of target cells in the test culture compared with the reference culture, and these differences could be amplified such that at the time of the pulse the number of target cells in the two cultures is different. Therefore, differential kinetics would violate the calculation of instantaneous suppression, which requires cultures with equal numbers of target cells. Another possibility, perhaps biologically more interesting, is that the regulatory cell population expands when cocultured with their targets, increasing the ratio *R/A*. Indeed, the data can be easily fitted if the number of regulatory cells increases ~6- and 10-fold, respectively, in the cases of infinite and equal affinities of regulatory cells. Because it is well established that regulatory T cells do not proliferate when stimulated with APCs in the absence of target cells, it is not trivial that the regulatory population will grow when cocultured with target cells. However, there are two non-mutually exclusive mechanisms that could explain this target cell-dependent growth of the regulatory cell population. The regulatory cells may promote differentiation of the target cells into a regulatory phenotype, such that the regulatory population would increase by autocatalytic recruitment of new members. Regulatory populations in the systems studied here are made of anergic, or naturally anergic-like, cells. The proposition that anergic cells may render other cells anergic is not new and has been discussed in the context of immunosuppression and tolerance (26). Alternatively, the size of the regulatory cell population may increase during coculture with target cells, because the later produce a growth factor. The hallmark of anergic-like regulatory cells is the expression of high levels of IL-2R and the lack of secretion of IL-2. Therefore, regulatory cells are fully responsive to the IL-2 produced, which is responsible for the growth of target cells. Explicit modeling of the growth kinetics of regulatory and target cell populations using a model described previously (34), demonstrated that these two alternative explanations can easily account for the data (results not shown). The fitting is degenerated and dependent on several parameters, such as inhibitory and stimulatory coefficients, growth rates of regulatory and target cells, etc., which cannot be measured independently. Overall it is fair to conclude that the impact of the kinetics in the suppression index deserves a proper theoretical analysis. In a complementary approach, it would be worth developing immunosuppression assays in which the assumptions underlying instantaneous suppression would be controlled and validated. Ideally the thymidine incorporation pulse could be performed within the first hours of culture, before an eventual accumulation of kinetic effects. Alternatively, early effects of regulatory cells on target cells might be measured instead of the rather late inhibition of proliferation. Good candidates are the inhibition of expression of early activation markers such as CD69.

Finally, the instantaneous inhibition index may fail to account for the experimental data because the suppression is not strictly dependent on the formation of multicellular conjugates. At the initial proportions of the three cells in culture the probability of formation of multicellular conjugates becomes much too improbable. Therefore, any mechanism of suppression that requires recognition of the same APC for activation of both regulatory and effector cells, but in which the actual mechanism of suppression is operative even when that conjugation with APC is asynchronous, will be more efficient and would more easily fit the data. Recently, Thornton and Shevach (24) suggested that $CD25^+$ T cells following activation by APCs might nonspecifically suppress their targets by a mechanism that is no longer APC dependent. Although the underlying mechanism was not detailed, it may be more efficient than that explored here because it does not require multicellular conjugates, but, instead, proceeds by sequential pairwise conjugations of the regulatory first with the APC and then with target cells. However, this proposal is difficult to reconcile with previous reports demonstrating the requirement for linked recognition of the same APC, and also with the fact that suppression does not occur if cells are activated using plate-bound anti-CD3 (22, 23). In agreement with the linked recognition is the alternative hypothesis that regulatory cells may suppress their targets indirectly by modulating the APCs themselves (28, 29). This mechanism can be expected a priori to be more efficient because it involves sequential interactions of the APC first with regulatory cells and then with target cells. Interestingly, the model as formulated here represents a minimal implementation of this mechanism, assuming that the effect of regulatory cells on the APC is very transient. Much higher efficiencies can be achieved if each regulatory cell is able to serially conjugate with different APC, permanently inactivating their capacity to stimulate target cells. Recent reports have shown that $CD25^+$ T cells or anergic cells can modulate their APCs (27, 30); however, this mechanism remains difficult to reconcile with the fact that significant suppression is scored in the presence of fixed or irradiated APCs (44), which cannot be modulated. A comparison of the capacities of these alternative hypotheses to explain the experimental data is being conducted.

The results reported here suggest that the population of target cells might promote growth of the population of regulatory cells in vitro. A generic analysis of the population dynamics of regulatory and target cell populations involved in adoptive transfers of tolerance leads us to a similar conclusion (34). These two complementary lines of evidence indicate that the maintenance and growth of a regulatory population might depend on the target population it regulates. It is tempting to suggest that potentially pathogenic cells, which are the targets of tolerogenic regulatory cells, may actually play an active role in the maintenance of tolerance.

In summary, our quantitative analysis of the mechanism of linked suppression dependent on multicellular conjugates allowed us to predict that it is mainly determined by the ratio of regulatory cells per APC and to accurately estimate a limit in efficiency of the suppression. Analysis of experimental data indicates that experimental suppression is mainly determined by the ratio of regulatory cells per APC, as expected from this model. Although having limited efficiency, an active inhibitory signal delivered by regulatory to target T cells in multicellular conjugates with a single APC can still explain the data if the number of regulatory cells increases in cocultures with target cells or if only a small fraction of APCs form conjugates.

Acknowledgments

We thank Prof. Robert Lechler, Prof. Shimon Sakaguchi, and Dr. António Bandeira for discussions from which this work originated. We also thank João Sousa, Jose Faro, and Jocelyne Demengeot for many useful discussions and for critically reading this manuscript.

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Target CD4+CD25- T cells promote the growth of regulatory CD4+CD25+ T cells in vitro.

Vanessa Oliveira[3]*, Kalet Leon[3]*†, Íris Caramalho[3]*, Shohei Hori*, Matthias Haury*, Jocelyne Demengeot*, Jorge Carneiro*[2]

* Instituto Gulbenkian de Ciência, Oeiras, Portugal. † Centro de Inmunología Molecular, Habana, Cuba.
[3]These authors contributed equally to this work. [2]Correspondence author.

Abstract

Regulatory $CD4^+CD25^+$ T cells are involved in the prevention of autoimmunity, allograft acceptance and T cell homeostasis. These cells have been shown to control the proliferation of $CD4^+CD25^-$ lymphocytes *in vitro*, however their mechanism of action and dynamics remain unclear. Assuming that suppression is dependent on multicellular conjugates with APC, modelling of the dynamics of regulatory and target cells lead us to propose a target cell-dependent growth of regulatory cells. In the present work, we further demonstrate that proliferation of regulatory cells as a function of an interaction with target cells during *in vitro* suppression assays may increase to realistic levels the otherwise inefficient suppression in multicellular conjugates. We tested our theoretical predictions experimentally by monitoring the proliferative history of individual cells using CFSE-labelling. We show that regulatory $CD4^+CD25^+$ T cells, which are unable to proliferate when stimulated alone, expand when stimulated together with target $CD4^+CD25^-$ T cells. Moreover, proliferation of regulatory cells directly correlates with expansion of target cells. These results suggest that a common growth factor controls the expansion of both cell populations, and that its production is specific of the target cells and inhibited by regulatory cells. The significance of these findings for our understanding of regulatory T cell phenomenology and the potential nature of the growth factor are discussed.

Introduction

Regulatory $CD4^+CD25^+$ T cells have been implicated in prevention of autoimmunity (1, 2), in dumping pathogenic inflammatory reactions triggered by microorganisms (3-5) and in controlling the size of the remaining $CD4^+$ T lymphocyte pool (6, 7). Despite the fundamental and clinical significance of these cells little is known about their origin, population dynamics and mechanism of action.

Several observations suggest that regulatory cells are generated in the thymus throughout life (8, 9), and may be selected upon interaction with high affinity/avidity ligands (10). Regulatory cells able to prevent autoimmune thyroiditis can be found in animals with an intact thyroid but not in animals where this organ has been ablated (11). These observations indicate that once in the periphery, persistence and expansion of regulatory cells appears to require recurrent interactions with APCs expressing specific TCR-ligands. Intriguingly, purified regulatory $CD4^+CD25^+$ T cells fail to proliferate when stimulated with a TCR-trigger and APCs *in vitro* while exogenous IL-2 can drive them into cell cycle, suggesting that they are unable to produce autocrine growth factors (12, 13). Finally, upon adoptive transfer in lympho-deficient animals, $CD4^+CD25^+$ T cells can expand in vivo (7). It is therefore probable that in vivo, regulatory T cells have access to growth factors in addition to ligand and APCs.

Attempts to dissect the mechanism of action of regulatory cells demonstrated that they suppress the proliferation of target cell *in vitro*, a process which seems to involve active inhibition of IL-2 expression (13). Most studies, with the exception of a recent controversial report (14), indicate that suppression requires direct cell-to-cell contact between antigen presenting cells (APCs), the regulatory cells and the other T cells that are targets of regulation (12). These observations suggested that suppression takes place while the regulatory cell and its target cell are co-conjugated with a single APC. Alternative mechanisms have been proposed, ranging from simple competition for conjugation with APC (15, 16), or nonspecific T cell–T cell interactions (14, 17), to transfer of signal from the regulatory cell to the target via the APC (18-20).

A mechanism of suppression dependent on multicellular conjugates with APC offers some degree of specificity by linked recognition (16) and has strong quantitative constraints, since its rate-limiting step is the encounter of the three cell types (21, 22). The plausibility of such a mechanism is testable by challenging its quantitative predictions. In a previous work we modelled the population dynamics of regulatory and target cells, assuming interaction as multicellular conjugates with the APC and therefore competition for APC accessibility (23). The major corner stone of regulatory cell phenomenology is that incidence of autoimmunity in recipients adoptively transferred with both regulatory and target cells depends on the relative and/or absolute size of two cell subpopulations (1). According to our modelling, this key observation can only be explain when assuming that growth and persistence of the regulatory subpopulation is dependent on the target cells they suppress (23). Moreover, the extent of the inhibition scored during *in vitro* suppression assays is only compatible with a strict dependence on formation of multicellular conjugates involving APCs, regulatory and target cells, if less than 20% of the APCs would be functional and/or the size of the regulatory cell population would increase when co-stimulated in the presence of target cells (24). Theoretical analysis of regulatory cells acting in multicellular conjugates with APC led therefore, via two convergent but independent lines of reasoning, to the conviction that target cells may act as growth factor for regulatory cells.

In the present report, this refined hypothesis is directly addressed. Quantitative analyses of cell growth kinetics in suppression assays show that first, one can safely reject that regulatory cells do not proliferate during the suppression assay, and second, it is plausible that regulatory cells proliferate as a function of an interaction with target cells. We address this prediction experimentally, following the proliferative history of individual cells using CFSE-labelling. We confirm that regulatory T cells, unable to proliferate when stimulated alone, divide when stimulated together with target cells. Moreover, on a per cell basis, the proliferation of regulatory cells is inversely proportional to the suppression of target cells, suggesting that the two cell types share a growth factor produced by target cells and whose synthesis is inhibited by regulatory cells.

Materials and Methods

Mice

C57BL/6 and C57BL/6-Thy1^a, Igh^a, Gpi1^a mice originally purchased from The Jackson Laboratories (Bar Harbor, ME) were bred under specific pathogen free conditions in our animal facilities.

Cell preparations and purification

Single cell suspension from spleen and lymph nodes (LN) were obtained by forcing the organs through a 100-µm nylon mesh in HBSS supplemented with 2% FCS (both from Gibco-BRL, Life Technologies, Gaithersburg, MD). Erythrocytes were lysed with a buffer containing 154 mM NH_4Cl, 14.2 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.2. Pooled LN and erythrocyte depleted splenocytes cells were stained with CD4-PE (clone RM4-5, PharMingen, Beckton Dickinson, San Diego, CA) and CD25-Alexa (PC61, produced in the laboratory, coupled to Alexa Fluor™ 488, Molecular Probes, Eugene, OR). $CD4^+25^-$ and $CD4^+25^+$ cells were sort-purified on a MoFlo High Speed Cellsorter (Cytomation Inc., Fort Collins, CO). Purity was routinely of 99 and 96 % respectively. $CD4^+25^-$ cell were obtained from congenic C57BL/6 Thy1.1 mice and $CD4^+25^+$ from C57BL/6 Thy1.2. In some cases, purified cells resuspended at $2.5x10^6$ cells/ml in serum free HBSS were stained with 5µM CFSE (Molecular Probes, Oregon, USA) for 5 min at room temperature. The reaction was stopped by adding FCS (20% final) and stained cells were washed once in RPMI supplemented with 10% FCS. APC were prepared from erythrocyte depleted splenocytes: after specific lysis of the T cells with anti-Thy1 mAb (produced in the laboratory from the J1J hybridoma) and complement (low-tox rabbit

complement, Cedar Lane, Ontario, Canada), cells were irradiated at 30 Gy.

Cell cultures

Suppression assays were of two types. In the "target-fixed assay" each wells contained $2.5x10^4$ target cells ($CD4^+25^-$) and either none (reference culture) or variable number of regulatory ($CD4^+CD25^+$) cells (test culture). In the "total-fixed assay", the total number T cells seeded per well was kept fixed ($2.5x10^4$ cells), and the proportion of regulatory and target cells varied. Each test culture had a matching reference containing the same number of target cells alone. All cultures were set in U shaped 96 well plates (Corning Coster, Cambridge, MA) containing $5x10^4$ γ-irradiated APCs per well and maintained for 3 days. Culture media was composed of RPMI 1640 containing glutamax I supplemented with 10% FCS, streptomycin (50μg/ml), penicillin (50U/ml), 2-mercaptoethanol (50μM), Hepes buffer (10mM, pH 7,3), sodium pyruvate (1mM) (all from Gibco-BRL) and 0.5 μg/ml anti-CD3 (2C11, produced in the laboratory).

Measurement of proliferation

On day 3, cultures were pulsed for the last 6 hours with [^{3}H]-thymidine (1μCi/well, Amersham Pharmacia Biotech, Uppsala, Sweden) and incorporated radioactivity measured. The experimental inhibition index (denoted here I) was obtained by dividing the cpm obtained in the test culture by the cpm monitored in the reference culture. Each culture was done in triplicate. For flow cytometric analysis, 8 replicated wells were pooled at day 3 and stained with either biotinylated anti-Thy1.1 (CD90.1 clone OX-7) or anti-Thy1.2 (CD90.2 clone 53-2.1), revealed with APC-streptavidin (all from Pharmingen), in PBS 2%FCS 0.02% azide. Samples were suspended in 1 μg/ml propidium iodide (Sigma, St. Louis, MO) to gate out dead cells and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA) using the CellQuest program (Becton Dickinson). CFSE-histograms were decomposed into Gaussians representing each generation using a Macintosh application (available upon request at http://eao.igc.gulbenkian.pt/ti/index.html), which uses the routine *"mrqmin"* described and documented in Press et al. (25) that implements a Levenberg-Marquardt method for non-linear fitting. Gaussian decomposition was also used to estimate the frequency of $Thy1.1^+$ and $Thy1.1^-$ subpopulations when the two populations did not segregate clearly (see example in Fig.4B). The precursor frequency, progeny fraction (i.e. the fraction of cells that divided at least twice), the apparent division time of generation i and the average apparent division time of the whole progeny, denoted respectively f, p and tp_i^a and tp^a were determined using the formulas:

$$f = \sum_{i=2}^{n}(N_i/2^i) \Big/ \sum_{i=0}^{n}(N_i/2^i) \quad (1)$$

$$p = \sum_{i=2}^{n} N_i \Big/ \sum_{i=0}^{n} N_i \quad (2)$$

$$tp_i^a = \Delta / i \quad \text{with } i>2 \quad (3)$$

$$tp^a = \sum_{i=2}^{N} tp_i^a \quad (4)$$

where n is the maximal number of generations, N_i is the number of cells in each generation i, and Δ is the duration of the culture. The precursor frequency f calculated after Givan et al. (26), and the remaining formulas are based on their rational of neglecting the first round of division.

Mathematical modelling

According to the model we proposed before (23) and illustrated in Fig. 1, the population dynamics of a mixture of regulatory cells, target cells and APC is described by the following set of ordinary differential equations:

$$\frac{dT_t}{dt} = \frac{1}{t_p} \cdot \sum_{i=1}^{s}\sum_{j=0}^{s} \alpha_{ij} \cdot i \cdot A_{ij}(A_0, T_t, R_t) \quad (5)$$

$$\frac{dR_t}{dt} = \frac{1}{t_p} \cdot \sum_{j=1}^{s}\sum_{i=0}^{s} \beta_{ij} \cdot j \cdot A_{ij}(A_0, T_t, R_t) \quad (6)$$

In these equations: A_0 is the total number of APCs in the cultures which is assumed to be constant; T_t is the total number of target cells at time t; R_t is the total number of regulatory cells at time t ; $A_{ij}(A_0,R_t,T_t)$ is the number of conjugates at time t that containing 1 APC, i target cells and j regulatory cells; tpis the average division time of stimulated (target or regulatory) T cells; and α_{ij} andβ_{ij} are interaction coefficients. The coefficient α_{ij} (andβ_{ij}) defines the probability that a target cell (a regulatory cell) proliferates after participating in a multicellular conjugate containing i target cells and j regulatory cells. The conjugates $A_{ij}(A_0,R_t,T_t)$ are calculated as:

$$A_{ij}(A_0, T_t, R_t) = A_0 \cdot H[i+j, T_t + R_t, sA, s] \cdot H[i, T_t, T_t + R_t, i+j] \quad (7)$$

with H defined as:

$$H[k,l,m,n] = \binom{k}{l}\binom{m-k}{n-l} \Big/ \binom{m}{n} \quad (8)$$

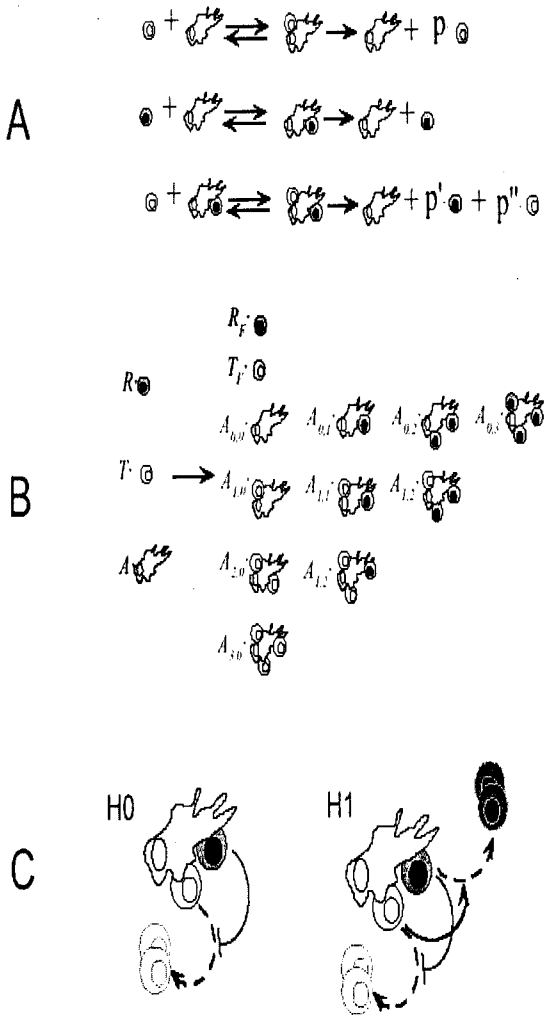

Fig 1. The ingredients of the model describing suppression of proliferation by regulatory T cells. A) Proliferation depends on productive conjugation of target and regulatory cells with the APC. The APC behaves as an enzyme with Michaelis-Menten kinetics, that catalizes T cell activation and proliferation. B) The progeny of a cell following productive conjugation and activation depends on interactions with other T cells simultaneous conjugated with the APC, and therefore on the stoichiometry of the multicellular conjugate. We follow the distribution of all possible multicellular conjugates which are in quasi-steady state at any given instant; C) Two alternative hypotheses about the interactions among conjugated target and regulatory differ in that regulatory cells are stimulated to proliferate by target cells according to H1 but not H0. In both hypotheses, the two cell types compete for conjugation with the APC, and regulatory cells abrogate the proliferation of target cells in the same conjugate.

In the equation above R_c and T_c are the total number of conjugated R and T cells at a given instant t, assuming that conjugation and de-conjugation are first order processes, that are practically in equilibrium.

$$K_T = \frac{T_c}{(T_t - T_c)(sA_0 - R_c - E_c)} \quad (9)$$

$$K_R = \frac{R_c}{(R_t - R_c)(sA_0 - R_c - E_c)} \quad (10)$$

These equations are used to model the results of *in vitro* suppression assays based on thymidine incorporation as described above. Assuming the amount of thymidine incorporated by a culture at a given time t is proportional to sum of the right-hand sides of eqns 5 and 6 (i.e. assuming that the period of the thymidine pulse is negligible), the inhibition index as predicted by the models is:

$$\lambda = \frac{\sum_{i=0}^{s}\sum_{j=0}^{s}(\alpha_{ij}\cdot i + \beta_{ij}\cdot j)\cdot A_{ij}^{test}(A_0, T_t^{test}, R_t^{test})}{\sum_{i=0}^{s}\sum_{j=0}^{s}\alpha_{ij}\cdot i\cdot A_{ij}^{ref}(A_0, T_t^{ref}, R_t^{ref})} \quad (11)$$

where the superscripts "test" and "ref" refer to the number of cells respectively in the test and reference cultures in the suppression assay at a given time t. For a given set of parameters, eqns. 5 and 6 are numerically integrated to obtain the number of regulatory and target cells in both reference and test cultures at time t, respectively R_t and T_t, based on the initial/input values R_0 and T_0. To compare with the experimental inhibition index obtained after a period Δ we compute $\lambda(A_0, R_\Delta, T_\Delta)$.

Results

Robustness of the inhibition index represented as a function of regulatory CD4+25+ T cells per APC.

Suppression of proliferation by regulatory T cells is generally measured *in vitro* by inhibition of [^{3}H]-thymidine incorporation. This assay involves two independent cell cultures with different cell densities: the reference culture contains APCs and target cells, while the test culture, includes in addition a given number of regulatory cells. On a per cell basis, proliferation measured by [^{3}H]-thymidine incorporation is known to be sensitive to cell density. Thus part of the inhibition observed by adding regulatory cells could be a spurious effect of the corresponding increase in cell density. We had noted before that the inhibition index is mainly dependent of the initial ratio regulatory cells per APC, and rather insensitive to changes in target cell numbers (24). This, in turn, is theoretically expected if inhibition is mainly due to an active interaction in multicellular

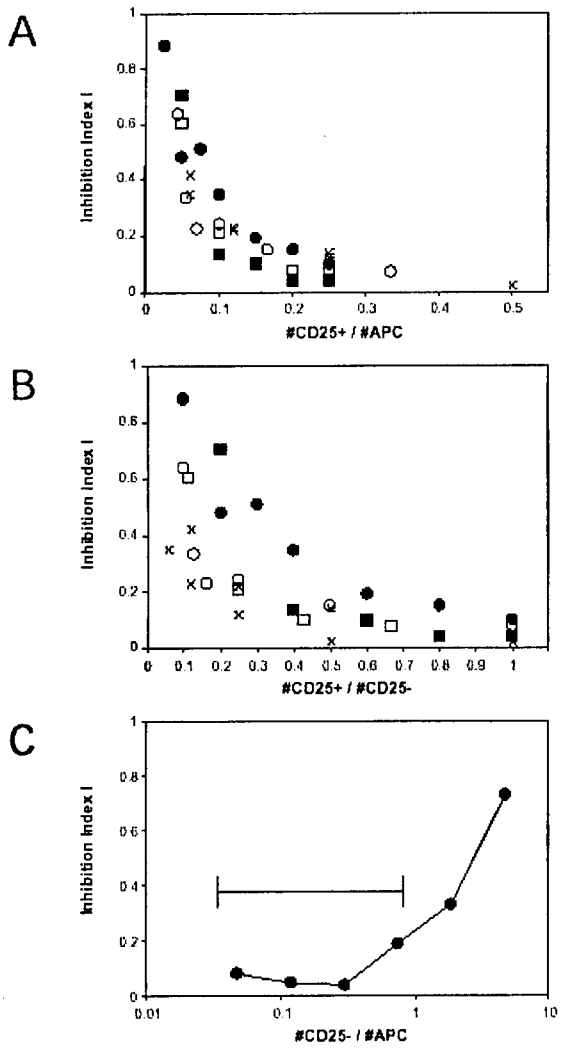

Fig 2. Robustness of a inhibition index I represented as a function of regulatory CD25+ T cells per APC. A and B) Inhibition index I was plotted against the seeding number of regulatory CD25+ T cells per APC R0/A0 (A) or against the seeding number of regulatory CD25+ cells per APC R0/T0 (B). The squares and circles represent two independent experiment using "total-fixed" (white) or "target-fixed" (black) assays. A0=$1x10^5$ cells, total fixed R0+T0=$5x10^4$ cells, and target fixed T0=$2.5x10^4$ cells. C) Inhibition index I in an assay where increasing numbers of target CD25- cells T0 were used to seed test/reference cultures with A0=$5x10^4$ cells and R0=$1.3x10^4$/0 cells. The line indicates numbers of target cells per APC that have been used in the experiments represented in A and B.

conjugates and not by competition for APCs. We directly assessed the effect of cell density on inhibition index by comparing two types of suppression assays. In classical "target-fixed" assays, the target cell ($CD4^+25^-$) number was kept constant in test and reference cultures, and increasing numbers of regulatory cells ($CD4^+25^+$) were titrated into test cultures. In "total-fixed" assay, the total T cell input to the test culture was kept constant and the ratio of regulatory cells per target cells increased. Each test culture was matched by a reference culture containing the same number of target cells and APCs, allowing to calculate the inhibition index I. If cell density would be playing a detectable role in our culture conditions then target- or total-fixed cultures should give significantly different results. In contrast, if the role of cell density is negligible and suppression is insensitive to differences in initial target cell number, the inhibition index scored in target-fixed and in total-fixed cultures should be identical for the same ratio of regulatory cells per APC. The inhibition index for each culture condition, after [^{3}H]-thymidine incorporation, was determined as described in Material and Methods. As depicted in Fig. 2A, when plotted as a function of the ratio regulatory cells per APC, the inhibition index values are identical independently of the type of assay. Moreover, multiple data sets produced by ourselves or others (12, 13) merge in a regulatory cells per APC coordinates but not in classical regulatory cells per target cell coordinates (Fig. 2A and B). Notwithstanding this fact, cultures containing increasing input of target cells and constant number of regulatory cells and APCs show a release of suppression (Fig. 2C). The inhibition index is therefore not absolutely insensitive to changes in target cell number, suggesting that some inhibition may be due to competition for APCs (24). Nevertheless, in the range of variation covered by our two culture conditions and those of others (12, 13), the initial target cell number has negligible or small impact on the inhibition index, which systematically appears within a narrow band when plotted as function of regulatory cell per APC.

High efficiency of suppression may reflect increased number of regulatory cells during co-cultures.

We argued before (24) that if suppression is strictly dependent on the formation of multicellular conjugates, the efficiency observed during *in vitro* suppression assays could be explained if the regulatory cell population expand during the co-cultures. This argument stemmed from a theoretical analysis of *in vitro* suppression assays in an ideal case in which proliferation could be measured instantaneously at the time point when cells are put into culture. However, two types of kinetic effects could invalidate our previous argument. First, since cells grow exponentially when cultured *in vitro* and suppression implies lower per capita growth rates, the ratio between

total proliferation in test and reference cultures (inhibition index *I(t)*) should decrease exponentially with time. This implies that any value of the inhibition index can be reached if the cultures are maintained long enough, even for conditions in which the number of regulatory cells per APC is very small. Second, the proliferation of the regulatory cells is a two-edged sword: it increases inhibition by increasing the number of regulatory cells per APC, and it increases the score of thymidine incorporation in the test culture. To directly evaluate these kinetic effects we compared the working hypothesis (H_1) that regulatory cells proliferate when co-cultured and stimulated with target cells, with H_0 – the negation of H_1 – that assumes that regulatory cells never proliferate even when stimulated in the presence of target cells. To conclude in favor of the working hypothesis, H_0 must be rejected. We therefore explored H_0 in the ideal parameter regime that maximize suppression.

In our model H_0 is specified by setting the coefficients of interaction α_{ij} and β_{ij} as follows:

$$\forall_{ij\neq 0}: \alpha_{i0}=1,\ \alpha_{ij}=0,\ \beta_{ij}=0 \qquad (12)$$

where α_{ij} and β_{ij} are the proliferation probabilities of each target cell and each regulatory cell respectively, that participate in a conjugate with i target cells and j regulatory cells. According to eqn 11 a single regulatory cell will abrogate the proliferation of all target cells participating in the same multicellular conjugate. Suppression is also increased if one assumes that, first, the conjugation constant of target cells is high; second, the regulatory cells are permanently conjugated with APC ($K_T/K_R=0$); and third, the number of sites per APC is practically infinite ($s>>(R_t+T_t)/A_0$). Relaxing any of these assumptions decreases suppression. Finally, the remaining parameter is the average division time t_p of the stimulated target cells. The shorter the division time of target cells the lower the predicted inhibition index λ (Fig 3B). Values of tp shorter than 6 hours bring the predicted index λ within the range of experimental observations I.

Assessing how probable these division rates are requires a probability distribution of the average cell division time in standard conditions of the immunossuppression assay. Since this distribution is not available we produced a CFSE-profile of T cells stimulated for 3 days under optimal conditions for proliferation (which in our hands corresponded to use an excess of live APCs and anti-CD3) (Fig3A-left). Using this profile we estimated a cumulative frequency distribution for division times F(tp) (Fig.3A-right). Briefly, for each peak i in the CFSE-profile we calculated the average apparent division time tp_i^a (eqn. 3) and the corresponding frequency of cells (neglecting cells that did not divide). This allowed us to calculate the frequency of cells $F(tp_i^a)$ that under best conditions divided on average in less than tp_i^a. This distribution will overestimate the frequency of cells responding with less than tp and can thus be used to assess the plausibility of the null hypothesis H_o. The CFSE-profile can be regarded as the result of distinct subpopulations of cells which differ in intrinsic division times. The cumulative frequency distribution (Fig 3A-right) would therefore represent the division times distribution of cells optimally-stimulated *in vitro*. The predictions of the model for different tp values, under the assumptions listed above that maximize suppression, are presented in Fig. 3B. The numbers in brackets are the frequency of cells that proliferated in less than tp when stimulated in optimal conditions. The predicted curves able to explain the experimental values correspond to unlikely values of tp (frequencies lower than 0.01). Alternatively, the CFSE-profile can be regarded as the result of an homogeneous population whose division time is distributed due to some stochastic process (e.g. encounters between cells). Under this assumption and these optimal conditions, the T cell population divides on average with $tp \trianglelefteq 2$hours. For these tp values, the prediction is clearly outside the range of the experimental data (heavy line on Fig 3B). According to these two considerations we can therefore safely reject the hypothesis H_0, and exclude that regulatory cells do not grow in co-cultures.

We then tested the alternative hypothesis H_1, according to which regulatory cells are stimulated to proliferate as function of a paracrine growth factor secreted by target cells after productive conjugation with an APC (i.e. produced by conjugated and not-suppressed target cells). This is modelled by specifying the interaction coefficients as:

$$\forall_{i\geq 0, j\geq 1}:\ \alpha_{ij}=\frac{1}{1+\omega\cdot j},\ \beta_{ij}=\frac{\chi}{\chi+c\cdot A_0} \qquad (13)$$

where: ω is the number of regulatory cells needed in an individual conjugate to achieve half-maximal inhibition of the target cells in the same conjugate; χ is the sum of all target cells that at each instant are conjugated and not-suppressed:

$$\chi=\sum_{i=1}^{i}\alpha_{ij}A_{ij}(A_0,T_t,R_t) \qquad (14)$$

and c is a constant that defines the number of conjugated not-suppressed target cells per APC required for half-maximal proliferation of regulatory cells. Moreover, the

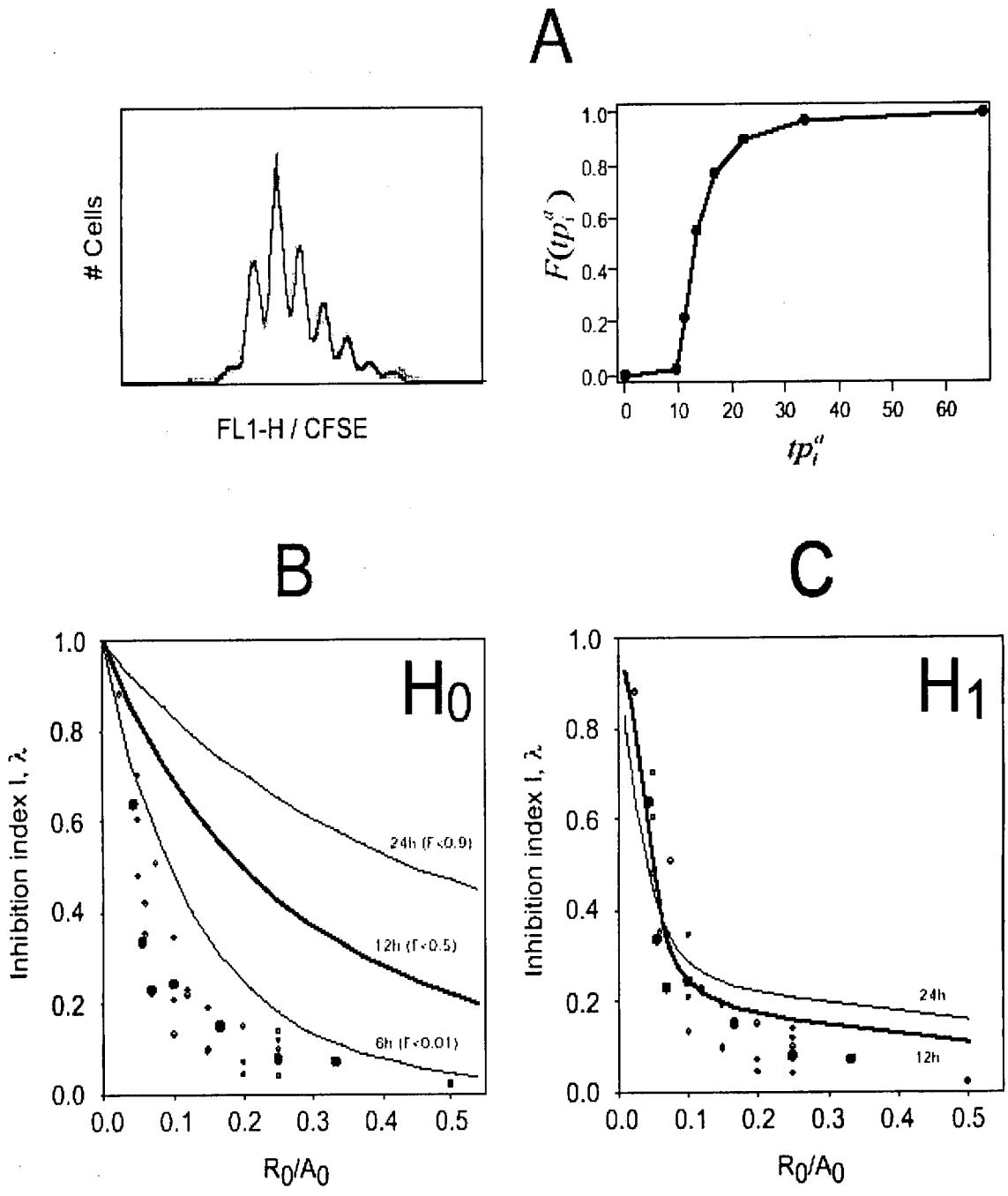

Fig 3. Suppression dependent on multicellular conjugates can explain the efficiency of suppression in vitro if regulatory cells are stimulated to proliferate by target cells (H1) but not if regulatory cells never proliferate (H0). (A) A profile of CFSE-fluorescence intensity of T cells stimulated with excess of live APCs for three days (left) is used to obtain an empirical cumulative frequency distribution of doubling times (right); (B) The model parameterised according to hypothesis H0 under conditions that maximize the efficiency of suppression for different division time was used to predict the dependence of the suppression index I as a function of the number of regulatory T cells per APC in the beginning of the culture (R0/A0). The numbers in brackets are the probability according to the empirical distribution. The symbols are the experimental results from fig. 2 (circles are our own data and crosses are published data); (C) The Model is parameterized according to hypothesis H1 and plotted for the average division time of tp=12 hours or tp=24 hours (respectively the average from B and from table 1)

definition of the coefficient β_{ij} assumes that the processes of secretion of the paracrine growth factor and its cell independent decay are practically in equilibrium. Note that this functional form of β_{ij} is appropriate because it is zero when the number of target cells is zero, i.e. there is no proliferation of regulatory cells when they are stimulated alone. This model is able to explain the data under many different parameter sets. This is illustrated in Fig 3C where different theoretical curves of λ are obtained by fixing tp to the indicated values, and tuning the remaining parameters such that these curves cross the experimental data points at half-maximal inhibition (i.e. $I=\lambda=0.5$) (for parameters values see figure legend). Structurally the model has some limitation namely that it is difficult to reach some extreme low values of the inhibition index. This limitation is due to the simple assumption that cells do not die. Relaxing this assumption can lead to much better fittings, with theoretical curves that always lay within the band defined by the experimental data (unpublished studies).

In conclusion, we explored H_0 in ideal parameter regimes that maximize suppression and show that it cannot explain the experimental data. We also show that H_1 can explain the data under realistic and broad parameter regimes. Taken together, these results strongly suggest that regulatory T cells do proliferate when co-cultured with target cells.

Regulatory $CD4^+25^+$ cells do expand while inhibiting the proliferation of $CD4^+25^-$ target cells.

Following the theoretical results from the previous section, we investigated experimentally whether regulatory $CD25^+$ T cells do indeed proliferate when co-cultured with their $CD25^-$ targets. We measured the proliferation of each cell population after coculture by monitoring their specific decrease of CFSE labeling. The $CD4^+25^+$ and $CD4^+25^-$ cells were purified from C57Bl/6 and congenic animals, expressing the Thy1.2 and Thy1.1 molecules respectively (Fig.4A). The $Thy1.1^+CD4^+25^-$ and $Thy1.2^+CD4^+25^+$ cells were labeled with CFSE prior to setting a "total-fixed" *in vitro* suppression assay. After three days of culture the profiles of CFSE staining specific for the target and regulatory lymphocytes was established using discriminatory anti-Thy1.1 and Thy1.2 staining (Fig. 4B).

In parallel, the same cultures were pulsed with [^{3}H]-thymidine and the inhibition indexes when plotted against the number of $Thy1.2^+CD4^+25^+$ cells per APC (Fig. 2A and Fig. 3) fall in the narrow band identified in the previous section. Measuring the individual progeny of $Thy1.1^+CD4^+25^-$ cells we confirmed that the maximal proliferation was observed when these cells were stimulated with APC and anti-CD3 in the absence regulatory cells. In the experiment depicted in Fig. 4C this maximal extent of proliferation leads to a progeny fraction p =90%, a frequency of responders f=61%, and an average apparent division time tp^a =21h. In co-cultures and as expected from concomitant measures of thymidine incorporation there is a progressive inhibition of the proliferation as the proportion of target cells:regulator cells increases (Fig.4C and Table 1). Hence, our analysis reveals for the first time that regulatory cells affect the percentage of responder cells inside the $CD4^+25^-$ population and interfere only marginally with their apparent division time (tp^a =23h).

In line with previous observations, the regulatory cell population does not proliferate when cultured with APCs and anti-CD3 in the absence of target cells. This is revealed by an amount of thymidine-incorporation that is not significantly higher than the background (not shown) but also by the presence of a single peak in CFSE-histograms, which is indistinguishable from the control non-stimulated cells. Strikingly, and as predicted on theoretical grounds, the CFSE-histograms specific for regulatory cells upon co-cultures show readily detectable additional peaks, demonstrating that in the presence of target cells, regulatory cells do proliferate (Fig. 4C and Table 1). Moreover, the extent of their expansion clearly increases as the proportion of target:regulatory cells increases (Fig. 4C and Table 1). The progeny fraction rose up to p=66% and the frequency of responders to f=21% in cultures set at an 80:20 ratio of target:regulatory cells. Remarkably, these values are similar to those obtained for the target $CD4^+25^-$cells in the same cultures conditions (Table 1). This last result, together with the fact that the proportions of Thy1.1:Thy1.2 cells remained constant or became biased towards Thy1.2 (Table 1) exclude that the proliferation measured within the Thy1.2 population would be due to a contaminating sub-population of $CD4^+25^-$ cells.

The actual progeny and precursor frequencies for each cell population varied considerably across experiments Fig 5), although the initial number of live cells in each set of cultures was kept constant. We attribute these variations to the uncontrollable toxicity of the CFSE, which may results in considerable variations in number of potential responder seeded in separated experiments. Although not in the direct topic of this

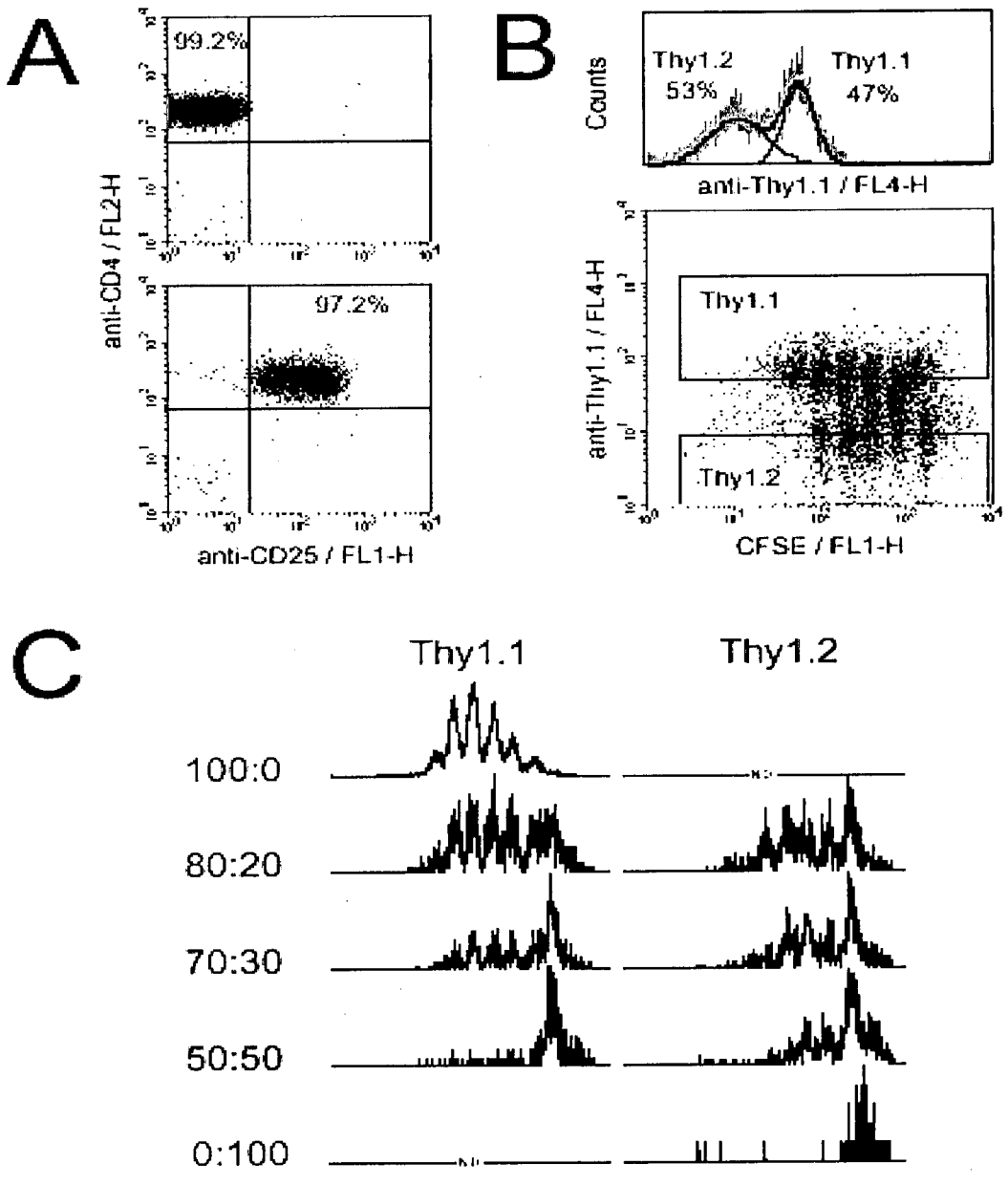

Fig 4. Regulatory Thy1.2+CD4+25+ cells do expand while suppressing Thy1.1+CD4+25- target cells proliferation. A) Purification of regulatory and target cells B) Gates for quantification of percentages of Thy1.1+ and Thy1.2+ (Thy1.1-) and the progeny in each generation after 3 days of co-culture. In situations, as the one illustrated in which distribution of Thy1.1 and Thy1.1- populations overlapped, the relative frequency was quantified by deconvolution into Gaussians (top) and the gates for CFSE-histogram included only the non-ambiguous halves of the Gaussians (bottom). C) CFSE-intensity histograms for the Thy1.2 (Thy1.1-) and Thy1.1 (+) cells after 3 days of co-culture. The numbers on the left are the original proportions of Thy1.1 and Thy1.2 cells seeded in the culture.

Table 1. Expansion of regulatory $CD4^+CD25^+$ in co-culture parallels the growth of target $CD4^+CD25^-$ cells.

Original Proportions Thy1.1:Thy1.2:APC	Proportion Thy1.1:Thy1.2 After 3 days	Target $CD4^+25^-$ cells ($Thy1.1^+$)			Regulatory $CD4^+25^+$ cells ($Thy1.1^-$)		
		F	P	tp^a (hours)	F	p	tp^a (hours)
100:0:200	—	61%	90%	21	—	—	—
0:100:200	—	—	—	—	0	0	>72
80:20:200	47:53	19%	65%	23	21%	66%	22
70:30:200	37:63	11%	48%	23	21%	60%	26
50:50:200	24:76	2%	13%	23	12%	36%	25

Precursor frequency (f), progeny (p), and average apparent division time (tp^a) were evaluated as described in Material and Methods from the experimental data displayed in Fig.4

work, we find worth reporting that the major cell death due to CFSE staining in our hands, occurs in the following 18 hours. In the latest experiments (Fig.4C and black circles in Fig. 5), we therefore set the suppression assay after an overnight incubation of stained cells in media alone. Most importantly, in independent co-cultures and irrespectively of the extent of cells proliferation, the growth of regulatory and target cell populations is comparable (Fig 5). A direct consequence of this dynamic is therefore that the lesser the suppression the higher the extent of proliferation for both populations (Fig. 5). This result, in turn, strongly suggests that regulatory cells and target cells share a growth factor which production is insured by the latter and inhibited by the former.

Finally, the average apparent division time of the two populations, tp^a =21-25h (Table 1), indicates that the true division time could be in a range ($12h \le tp < 21-25h$) where the model explains the inhibition index values determined in the parallel [^{3}H]-thymidine assays (Fig. 3). In summary, we show that regulatory $CD4^+25^+$ cells do proliferate when stimulated in the presence of target $CD4^+25^-$ cells, and that this finding could per se explain the high inhibition index found by classical approaches.

Discussion

In this article we provide the empirical demonstration that regulatory $CD4^+25^+$ T cells grow when co-cultured with target $CD4^+25^-$ T cells. This result may have significant implications for the understanding of the relationships between the molecular phenotype, the mechanism of action, and factors determining the persistence and expansion of regulatory T cells in the periphery.

The experimental observation that regulatory cells proliferate as a function of the target cells per se does not establish a causal link between the expansion of the regulatory population and the extent of suppression. Proliferation could be just a side effect of a growth factor produced by target cells but remaining unrelated to the efficiency of suppression. The analysis of "instantaneous suppression" we provided before (24) and the present analysis of the kinetics of growth during suppression assays indicate that the ratio regulatory cells per APC can increase and in so doing explain the extent of suppression observed. The mathematical model appears here as a tool to reveal a putative causal relationship that is otherwise difficult to derive experimentally. Direct experimental demonstration that the extent of suppression observed requires first, minimal number of regulatory cells per APC, and second, expansion of regulatory cells remains, however, to be established. The first point can be assessed by titration as described in the data section, and such titration reveal that there is a culture condition where the inhibition index is insensitive to the number of target cells and depends exclusively on the number of regulatory cells per APC. To address the second point requires assays in which proliferation of regulatory cells can be specifically inhibited without interfering with their "biological integrity" neither with the proliferation of target cells. Although an ideal experimental design is still to be conceived, our preliminary results indicate that irradiated $CD4^+25^+$ cells, incapable of cycling, do not affect proliferation of normal $CD4^+25^-$ in classical suppression assay (not shown). Since irradiation may interfere with other cellular functions in addition to inhibit DNA replication, this experiment is not conclusive but it is nevertheless consistent with the hypothesis.

It has been argued that a mechanism of interactions between cells dependent on multicellular conjugates with

APC is much too inefficient to be functionally relevant in situation of T cell mediated suppression (27). However, our theoretical approach, shows that we can not disprove this mechanism on the basis of efficiency. Although in our model the suppression of target cells has an absolute limit in terms of the number of regulatory cells per APC (24), the inefficiency of the mechanism can be compensated by the dynamics of the interacting cell populations. We show here that the growth rate of T cells is enough to increase a much too low number of regulatory cells per APC to a number of regulatory cells that can explain experimental suppression. A similar situation could be taking place in vivo in peripheral organs, where clonal size regulation of autoreactive regulatory and target cells by one another, could guaranty maintenance of tolerance to self-components. Thus, in contrast to the other candidate mechanisms discussed above (14, 28), the hypothesis of suppression in multicellular conjugates, by correlating activation of target and regulatory cells in space and time, offers a reasonable degree of specificity.

Our results indicate that during co-cultures with $CD4^{+}25^{-}$ T cells, regulatory $CD4^{+}25^{+}$ T cells receive a growth factor that is otherwise unavailable. What might be the factor or factors involved in target cell-mediated proliferation of regulatory cells? The most straightforward candidate is IL-2. Regulatory cells grow in the presence of IL-2 (12, 13) and can even be cloned under these conditions (Sakaguchi, personal communication). Moreover, expressing the high-affinity receptor ($CD25^{+}$) they can potentially respond to lower concentrations of IL-2 than $CD25^{-}$ cells. Although the nature of the suppressive signals delivered from regulatory cells to target cells remain to be established, they result in the repression of the IL-2 gene expression (12, 13). The inhibition of endogenous IL-2 production in co-cultures is the most parsimonious explanation for a correlated proliferation of target and regulatory cells. Since proliferation of suppressed target cells can be restored by exogenous IL-2 ((12, 13), and our data not shown) it is unlikely that the suppressive signal would be transduced into other forms of inhibition than cell cycle progression. The axis represented by IL-2 induction and IL-2 responsiveness would therefore be at the core of the immunobiology of regulatory cells. This hypothesis would establish a connection between the population dynamics of regulatory cells, their molecular phenotype and their mechanism of action. Disturbances to tolerance and homeostasis observed in animals (29-32) or humans (33) mutant for molecules belonging to this axis could be partially interpreted as a loss of regulatory cells in the periphery (34-36), since in the absence of their growth factor this population would be unable to compensate for cell loss by death.

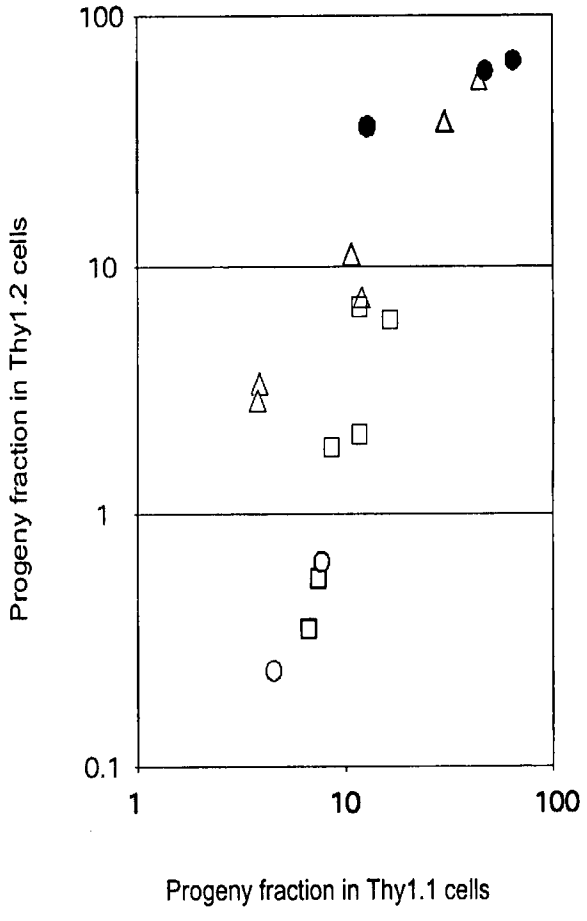

Fig 5. The proliferation scored by regulatory Thy1.2+CD4+CD25+ T cells and target Th1.1-CD4+CD25- T cells are correlated in co-cultures. The progeny of regulatory Thy1.2+ cells is plotted against that of target Th1.1+ cells in co-cultures. The different symbols correspond to independent experiments.

Elsewhere (23) we argued, based on theoretical principles, that the body of data on adoptive transfers of tolerance implies a bistable dynamic system, and that this is a natural expectation if the persistence and expansion of the population of regulatory cells is dependent on target cells. The present results indicate that this mechanism is not only favoured by such theoretical principles but is also supported experimentally. Although the actual molecular basis remains to be established, a mechanism in which regulatory cells, use as source of growth factor, the cells they prevent from mounting immune responses, is functionally sound. It offers a way by which a regulatory cell population can adapt its size according to the

response of their targets, and therefore allow for a robust control of autoimmunity. This model provides a simple explanation for the apparent "reversion" of phenotype (loss of CD25 expression) observed when purified $CD4^+25^+$ are transferred alone into empty recipients but not when these cells are co-transferred with growth-promoting $CD4^+25^-$ cells (7, 37). In the first case, regulatory $CD4^+25^+$ cells would be lost until a minor subpopulation of contaminating target $CD4^+25^-$ cells expands enough to sustain them. In the second case, the regulatory cells would readily persist and grow using growth factors produced by co-transferred $CD4^+25^-$ cells. However, the model is based on linked suppression involving interactions between T cells in multicellular conjugates. Since all T cells recognising peptides on the same APC are 'linked suppression' targets of regulatory cells (16), this raises a specificity issue. How can self-antigen driven regulatory T cells prevent autoimmunity and simultaneously allow for immune responses to foreign antigens? This seems not to be simply a theoretical curiosity, since regulatory cells do down-regulate immune responses to invading pathogens and allergens (5, 38). Addressing this issue requires extending our models, which assumed homogeneous populations of regulatory cells, to models in which there are multiple clones containing cells with regulatory and/or immune phenotype. Such studies are undergoing and should further our understanding of the role of regulatory cells in self-tolerance and in the prevention of deleterious immune responses.

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Tolerance and Immunity in a mathematical model of T cell mediated suppression

Kalet León[1, 2, 3], Agustin Lage[1] & Jorge Carneiro[2]

[1] Centro de Inmunología Molecular, Habana, Cuba. [2] Instituto Gulbenkian de Ciência, Oeiras, Portugal.

[3] Author to whom correspondence should be addressed.

Abstract

Regulatory CD4+CD25+ T cells play a major role in natural tolerance to body components and therefore are relevant to understand the self-nonself discrimination. The most pressing theoretical question, regarding the fact that these regulatory cells perform their function through linked recognition of the APCs, is how this "nonspecific" mechanism permits a proper balance between tolerance and immunity that is compatible with an effective self-nonself discrimination. To tackle this issue, we develop a numerical simulation, which extends a previous mathematical model of T cells mediated suppression to include the thymic generation and peripheral dynamics of many T cell clones. This simulation can mimic the capacity of the immune system to establish natural tolerance to self-antigens and reliably mount immune responses to foreign antigens. Natural tolerance is based on ubiquitous and constitutive self-antigens, which select and sustain clones of specific regulatory cells. Immune responses to foreign antigens are only achieved if they displace the self-antigens from the APCs, leading to a loss of the regulatory cells, and/or if the foreign antigen entails a sharp increase in the total number of APCs. Meaningful behavior requires that a minimum number of new T cells enter the periphery per unit of time, being their repertoire selected so that anti-self affinities are within a proper interval, but differentiation to regulatory cells may be antigen non-specific. We conclude that positive selection is required to generate a sufficiently high frequency of self-antigen specific regulatory cells that reliably effect natural tolerance. Negative selection is required to avoid the emergence at the periphery of very high affinity anti-self regulatory cells that will make the tolerant state so robust that it could no be broken by the introduction of a foreign antigen. This result highlights the importance of repertoire selection in dominant tolerance proposing a novel role for the processes of positive and negative selection within this frame.

1. Introduction.

Regulatory T cells, which are contained within the CD4+CD25+ pool and actively suppress the activity of other T cells, are gaining increasing relevance in Immunology (Shevach, 2000; Annacker et al., 2001a; Coutinho et al., 2001; Sakaguchi et al., 2001). Although the inability to fully isolate such regulatory T cell has been an obstacle to their characterization, an increasing amount of information is accumulating about their properties and function. It is now clear that regulatory T cells develop in the thymus (Heath et al., 1996; Itoh et al., 1999). The processes mediating the differentiation of thymocytes into the regulatory phenotype are unclear, although regulatory CD4+CD25+ T cells can be selected upon high avidity interactions with self-antigen recognition (Jordan et al., 2000; Jordan et al., 2001), as previously proposed on theoretical grounds (Modigliani et al., 1996). Notwithstanding, it is still unresolved whether the

repertoire of regulatory cells is differentially shaped in terms of self-antigen recognition and furthermore if differentiation to the regulatory phenotype is antigen-mediated. In the periphery, CD4+CD25+ T cells represent 5 to 10% of total CD4+ T cells, and are diverse in terms of Vbeta family (Takahashi et al., 1998) and CDR3 length of their TCR (Hori et al., 2002). This population can be maintained in the absence of thymic production (Annacker et al., 2001b), and the persistence of specific regulatory activity requires continuous antigenic stimulation (Seddon & Mason, 1999; Garza et al., 2000). In respect to the mechanism of action for these cells Waldmann et al reported, in a system in which T cell mediated tolerance is induced to sking grafts, that suppression requires that both tolerogenic and immune T cell populations recognize antigens expressed by the same antigen presenting cells (Cobbold et al., 1996; Davies et al., 1996; Wise et al., 1998). This type of "linked suppression" was further investigated in several in vitro systems (Takahashi et al., 1998; Thornton & Shevach, 1998) where, with the exception of a recent controversial result (Thornton & Shevach, 2000), the suppression mediated by regulatory T cells in vitro requires direct cell to cell contact between antigen presenting cells (APCs), regulatory T cells and T cells that are targets of regulation (Read et al., 1998; Takahashi et al., 1998; Thornton & Shevach, 1998). These two lines of evidence led to our current working hypothesis that suppression takes place when the regulatory T cell and its target T cell are co-conjugated with a single APC, i.e. suppression requires simultaneous interaction between three cell types in multicellular conjugates. Alternative hypotheses can be found in the literature, which range from simple competition for conjugation with APC (Lombardi et al., 1994; Cobbold & Waldmann, 1998) to more complex interactions in which the APC itself conveys a signal from the regulatory T cell to its target (Cederbom et al., 1998; Taams et al., 1998; Cederbom et al., 2000; Taams et al., 2000; Vendetti et al., 2000).

Suppression by CD4+CD25+ regulatory T cells has been clearly implicated in the prevention of autoimmunity (Sakaguchi et al., 1996), in the control of immune responses to pathogens (Powrie et al., 1997; Iwashiro et al., 2001; Singh et al., 2001), and in tolerance to allografts (Davies et al., 1999; Hara et al., 2001). Theoretically it has been claimed that these cells are major actors in Natural Tolerance (Modigliani et al., 1996; Sakaguchi et al., 1996) and therefore are relevant to understand self/non-self discrimination. So the most pressing theoretical question, regarding the observation that these cells perform their function through linked recognition of the APC, is how this protective mechanism still allows for normal immune responses to pathogens (Mason & Powrie, 1998). In other words, how this regulatory mechanism permits a proper balance between tolerance and immunity that is compatible with an effective self/non-self discrimination. Note that even under the extreme assumption that there are no regulatory T cells specific to foreign antigens, since foreign antigens are always co-presented with self-antigens by APCs, regulatory cells effecting linked suppression could prevent immune responses. Moreover to achieve an immune response to pathogens the previously established regulation to self-antigens is probably broken, and consequently it is unclear how it is reestablished after the effective pathogen elimination. The present work elaborates in these theoretical issues, resorting to mathematical modeling.

We have studied T cell mediated suppression by mathematical modeling the dynamics of Regulator cells, Effector cells, and APCs (Leon et al., 2000). We have compared different candidate mechanisms of the interaction between Regulators and Effectors in multicellular conjugates with APC, in terms of their capacity to explain observations in vivo (Leon et al., 2000) and in vitro (Leon et al., 2001). As a whole these studies suggested that Regulators actively inhibit proliferation of Effectors, while Effectors promote the growth of the Regulator population. Recently, were supported experimentally this expectation (Oliveira et al., 2002) showing that CD4+CD25+ regulatory T cells receive a growth factor from CD4+CD25- effector cells in vitro. Nevertheless, it is still possible that Regulators promote the differentiation of Effectors to the regulatory phenotype. In the present paper we develop a numerical simulator, which extends our previous mathematical models to include the thymic generation and peripheral dynamics of multiple clonal populations of Regulators, and Effectors T cells. We ask what are the conditions under which a realistic interplay between tolerance and immunity emerges from the dynamics of these cells populations.

2. The Model.

Our previous model described the evolution of density of two T cells populations, Effectors (E) and Regulators (R) that recognize a homogeneous and fixed population of APCs. Interactions between Regulators and Effectors require formation of multicellular conjugates (fig 1). In the present work, this model is extended to accommodate several subpopulations of Effectors (density E_i) and

Regulators (density R_i), distinguished according to their clonal antigen receptor (indicated by the index i) and to include a constant source of new randomly generated clones containing both Effectors and Regulators. The features of this extended model are described bellow.

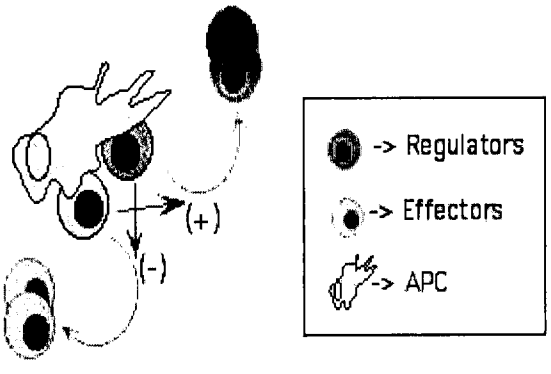


Fig.1- Cartoon illustrating the hypothetical mechanisms by which Regulators interact with Effectors when both cells participate in multicellular conjugates with the APC.

2.1 Compartments.

Two compartments are considered, representing the thymus and the periphery. New T cell clones are generated in the thymus, where they are selected and from where they are exported to the periphery. The periphery is modeled as a well-stirred compartment with fixed populations of APCs with density A. Interactions leading to activation, proliferation or suppression take place inside this compartment.

2.2 Antigens.

Two sets of antigens are distinguished. The antigens in the first set, denoted self-antigens, are always expressed both in the thymus and in the peripheral compartment. Unless state otherwise, the simulations reported here were performed using N_S=10 distinct self-antigens with different concentrations (Section 2.3). The simulations contemplate another antigen, called foreign-antigen, which initially is not expressed neither in the thymus nor in the periphery. This foreign-antigen is later "introduced" in the periphery, where its concentration is an arbitrary step function of time, corresponding to an instantaneous raise to a given value when it is introduced and an instantaneous decrease to zero when it is removed (Section 2.3).

In this study, differential expression of self-antigens in the thymus and peripheral compartments was not considered. The possibility that foreign-antigens are presented in the thymus was also neglected.

2.3 Antigen presentation and number of APCs

APCs express a fixed total number of MHC-peptides complexes. In reality processing and presentation of individual antigens results in several T cell epitopes, but for simplicity, each antigen is assumed here result in a single epitope. All the APCs in a compartment present the same set of L different epitopes/antigens. The relative epitope representation is the same for all APCs in the compartment, and is defined by a vector where the element p_l represents the fraction of MHC-molecules expressing the lth epitope/antigen. In the absence of foreign antigens the antigenic composition of the compartment is defined as L=N_S and $p_l = p_l^o$ satisfying:

$$1 = \sum_{l=1}^{N_S} p_l^o \qquad (1)$$

The antigenic composition is initially the same in the thymus and the peripheral compartment. A foreign-antigen is instantaneously introduced to the peripheral compartment by setting the local number of epitopes to L= N_S+1, and changing accordingly the fractional distribution of the self-epitopes on the APCs. Denoting by p_F the fraction of MHC-molecules occupied by the foreign epitope and assuming that the self-antigen epitopes are linearly displaced, the fraction p_l of the lth epitope after the introduction of foreign-antigen becomes:

$$p_l = \begin{cases} (1-p_F)\,p_l^o & if\ 1 \le i \le N_s \\ p_F & if\ i = N_s + 1 \end{cases} \qquad (2)$$

This linear dependence is a simple first approximation to the complex problem of antigen processing and presentation that appropriately captures the fact that introducing a new antigen will necessarily displace previously presented antigens. In some simulations the foreign-antigen is instantaneously removed later by re-setting the number of epitopes to L=Ns and redefining the fractions pl according to eqn. 1.

The two compartments, thymus and periphery, have a given basal density of APCs $A = A_0$. In most simulations the concentration of APCs in the peripheral compartment is kept constant $A=A_0$. In some simulations, however, the introduction of the foreign antigen is assumed to increase the concentration of APCs by a factor δ such that $A=(1+\delta)A_0$.

2.4 T cells APC conjugation.

The interaction between the cells of the clone i and the APCs is characterized by a conjugation constant Ki ((Leon

et al., 2000); Section 2.5), which is defined as the following function of the interactions between the clonal TCR and the L different epitopes presented by the APC.

$$K_i = K_m \frac{(\sigma_i)^h}{1+(\sigma_i)^h}\ ,\ \sigma_i = m\sum_{l=1}^{L} k_{i,l}\, p_l \qquad (3)$$

Where:

$k_{i,l}$ is the affinity of interaction of the TCR of T cell clone i with the epitope l.

p_l is the fraction of epitope l on the APCs.

K_m is the maximal value of the conjugation constant.

h is a coefficient that specifies the degree of cooperativity in the conjugation process.

m is the total surface concentration of MHC epitopes per APC, which is set to 1 for simplicity

Equation 3 assumes that the conjugation constant between APCs and T cells is a cooperative saturating function of the different TCR-MHC-peptide interactions. The T cell sums the signal resulting from the recognition of different epitopes on the APC. If there is too little signal the T cell will barely attach to the APC. Above a certain level of recognition by the T cells there is no gain in recognizing more peptides, since the cell will attach to the APC with a maximum conjugation constant Km

2.5 T cell population dynamic in the periphery.

The elementary dynamics of Regulators and Effectors in the peripheral compartment was described previously (see Model 3 in Leon et al. 2000). Qualitatively, it is assumed that the Regulators inhibit the growth of the Effector population, while the growth of the Regulator population is promoted by interactions with Effectors. The growth of the Regulators is either the consequence of their own Effector-dependent proliferation or a consequence of Regulator-dependent differentiation of Effectors that become Regulators. The equations describing a clone i containing both Effectors and Regulators are:

$$\frac{dE_i}{dt} = P_e \cdot Hyp[0, Rc^t \cdot V, s \cdot A \cdot V, s]\frac{sA}{sA - rc^t} Ec_i - \eta\,\mu\,\frac{s-1}{s}\cdot\frac{Rc^t}{A}\cdot Ec_i - d\cdot Ef_i \qquad (4)$$

$$\frac{dR_i}{dt} = \eta\,\mu\,\frac{s-1}{s}\cdot\frac{Rc^t}{A}\cdot Ec_i + (1-\eta)\,\mu\,\frac{s-1}{s}\frac{Ec^t}{A} Rc_i - d\cdot Rf_i$$

$$Hyp(N, N_0, M, L) = \binom{N_0}{N}\binom{M-N_0}{L-N}\Big/\binom{M}{L}$$

Where:

E_i is the concentration of Effectors of clone i

R_i is the concentration of Regulators of clone i

A is the concentration of APCs.

V is the volume of the peripheral compartment

t is the time variable

Ec_i, Rc_i is the concentration of conjugated Effectors and Regulators of clone i

$Ec^t = \sum_i Ec_i$ total concentration of conjugated Effectors

$Rc^t = \sum_i Rc_i$ total concentration of conjugated Regulators

$Ef_i = E_i - Ec_i$ is the concentration of free Effectors.

$Rf_i = R_i - Rc_i$ is the concentration of free Regulators.

s is the maximal number of conjugation sites per APC.

P_e is the po=roliferation rate of Effectors when stimulated by APC.

η is a switch variable controlling the mode of regulator populatioin growth: setting it to zero describes the mechanism where Regulators proliferate stimulated by Effectors and setting it to 1 describes the mechanism where the Effectors are induced to differentiate to the Regulators.

μ is the proliferation rate of stimulated Regulators if $\eta = 0$ or kinetic constant for Effector differentiation if $\eta = 1$.

d Death rate for T cells

Under quasi-steady states assumption, the concentration of conjugated Effectors or Regulators per APC are obtained respectively as:

$$Ec_i = \frac{F\,K_i}{1+F\,K_i}E_i,\quad Rc_i = \frac{F\,K_i}{1+F\,K_i}R_i \qquad (5)$$

Where K_i are the conjugation constant defined according to equation 3 and F is the number of free conjugation sites. The later is obtained by solving numerically the following equation.

$$F = sA - \sum_i \frac{F\,K_i}{1+F\,K_i}\cdot(E_i + R_i) \qquad (6)$$

The dynamics described by eqn. 4 represents two alternative mechanisms for the interaction between Regulators and Effectors in the model, corresponding to the values 1 and 0 of the "switch" parameter η . We have systematically studied these two alternatives models, but since the simulation results reported here were qualitatively equivalent in both models, we report only the case $\eta = 0$.

The number of parameters controlling T cell dynamic is reduced by fixing $P_e = 1.38\ day^{-1}$, $d = 0.23\ day^{-1}$ and s=5. Our reasoning here follows previous results (Leon et al., 2000) which demonstrated that once fixed P_e, d and s, any possible dynamical behavior of the model can be recovered by changing the values of parameter μ. So we decided to control the elementary dynamic in the simulation by parameter μ, fixing P_e, d and s to reasonable values. Note that these values correspond to a mean doubling time of E cells of 12 hours and life span of free T cells of 3 days. A more deep analysis of this parameter specification is done in section 3.1 in the results

2. 6 Metadynamic of T cell clones: influx from thymus and removal from periphery.

New T cells are continuously exported to the peripheral compartment after selection and education in the thymus (Section 2.6). Sc new clones, containing C cells per clone, enter the periphery per time step. Every new clone produced by the thymus is unique. This postulated can be justified given the almost unlimited potential diversity of the T cell variable regions (10^{16} (Davis & Bjorkman, 1988)) and has been commonly used in mathematical modelling in immunology (for instance in (Carneiro et al., 1996a; Carneiro et al., 1996b)). For every new clone i entering the peripheral compartment two new differential equations on Ri and Ei are appended to the system, describing the change in the number of Regulators and Effectors in the clone

A systematic study of the dependence of simulation results on parameters Sc and C allowed us to conclude that for sufficiently high values of Sc an asymptotic regime is reached where what matters is the product Sc.C (St). All the results reported here were obtained within this asymptotic regime and therefore we will only refer to St values. For implementation purposes, however, simulations were performed with Sc=1 new clone every 3 hours, such that the value St reflects a proportional change in C.

When the concentration of cells per APC in a clone i falls bellow a given value Tdead the clone is assumed to be extinct and the corresponding equations are removed from the list describing the compartment (de Boer & Perelson, 1991; Carneiro et al., 1996a). In all the simulations reported here the condition for removal was (Ei+Ri)/A<Tdead=0.05.

2. 7. Thymic selection and education.

The process of thymic selection is simulated according to the following algorithm. A new clone i is generated in the thymus. A pattern of antigen recognition is assigned following the method previously proposed by (Carneiro et al., 1996a; Carneiro et al., 1996b). Briefly, the probability that a given T cell clone recognizes a random epitope is assumed to be a constant recognition probability Pr. Given that a new clone i recognizes an epitope l, the affinity $k_{i,l}$ is drawn as random value in the interval $[k_{\min}, k_{\max}]$. Once all the pairwise affinities kil have been assigned the conjugation constant of the cells APCs expressing L=Ns epitopes is obtained according to eqn. 3. Those clones whose conjugation strength is outside the selection window represented by $[K_m \cdot W_{\min}, K_m \cdot W_{\max}]$ are eliminated. This process is iterated until Sc new clones are effectively generated per time step.

The number of parameters controlling the thymic selection process is reduced to a minimum by taking:

$$W_{\min} = 0.01 \cdot W_{\max},\ k_{\min} = \frac{m^{-1}}{0.7} \cdot \left(\frac{W_{\max}}{1 - W_{\max}} \right)^{1/h},$$

$$k_{\max} = \frac{m^{-1}}{0.001} \cdot \left(\frac{W_{\min}}{1 - W_{\min}} \right)^{1/h} \qquad (7)$$

The first condition guarantees that a sufficiently wide range of the conjugation strength to presented self-epitopes in the thymocytes is exported to the periphery (two order of magnitude seems reasonable). The second condition imposes that a self-epitope presented by more than 70 % of MHC-molecules will delete all the cells that recognize it. The third condition imposes that a self epitope representing less than a 0.1 % of total APC presentation cannot positively select a T cell clone unless this clone cross recognize another self-epitope. Note that by adopting eqn 7, a single parameter controls thymic selection in the model ($W_{\max}$).

Regulatory cells are generated in the thymus (Itoh et al., 1999; Jordan et al., 2000; Jordan et al., 2001) by an unknown process. In this work the differentiation of a thymocyte into Effector or Regulator is assumed to be independent of antigenic recognition. So in each clone generated, a fraction α are Regulators and a fraction $1-\alpha$ are Effectors. Note that this assumption, is in contrast with some hypotheses in the literature (Modigliani et al., 1996). In the results reported here we set $\alpha = 0.5$,

but the conclusions driven are valid for any other positive fraction.

In summary, at every time step, Sc new clones are appended to the list of clones in the peripheral compartment per unit of time. Each new clone i enters the periphery with concentrations Ri=αC/V and Ei=(1-α)C/V for Regulators and Effectors respectively.

2.8 Parameters of the model.

The simulation presented here has a considerable number of parameters (Table 1). Several parameter values have been fixed or constrained in the previous sections, were several radical simplifications were done. We remain with a relatively small "control" subset. Hence, the thymic output is controlled in magnitude by the parameter St and in "quality" of self-antigen recognition by parameter $W_{\max}$. The population dynamics in the periphery is controlled by the parameter μ, the concentration of APCs (A), the volume of the peripheral compartment (V) and the values of the conjugation constants for different clones which is modulated by parameter Km. The reactivity of the system to the introduction of a foreign antigen is controlled by parameter P_r, which give the probability that a T cells clone recognize any given antigenic epitope and by the parameter P_F, which set the relative level of presentation by APCs of the foreign antigen. The systematic study of these "control" parameters highlights some aspects of the behavior of the system and their (partial) parameter dependencies. A full analysis of all the parameters is not possible at this stage. Nevertheless, numerical studies have shown that changing the values of non-control parameters can change the particular quantitative values in the results, but does not alter significantly the qualitative behavior and dependencies of the model.

2.9 Implementation notes.

The simulation was coded in C-language. Simulations were performed in a PC Pentium-III 800 MGHz, 256 MGb of RAM, running Linux operative system. Numerical integration of ODEs was performed using a fourth-order Runge-Kutta method as described in Numerical Recipes in C (Press, 1997).

3. Results

3.0 Properties of the basic dynamical model.

We recapitulate here those results of our previous studies that are relevant to qualitatively understand the more complex simulations performed here. These simulations are an extension of a model studied before (Leon et al., 2000), where the growth of regulatory population is dependent on the effector population. This basic model, with three cell populations Effectors (E), Regulators (R) and APCs (A), has two possible stable steady states. In the first the Effectors out compete the Regulators, which go extinct, while in the second Effector and Regulator populations cohexist. We have previously interpreted the former state as immune reaction (immune or autoimmune state) and the latter state as tolerance. This model has three possible regimes illustrated in fig 2a . In the parameter regimes I and II, the model is globally stable always reaching the immune or the tolerant states respectively. In regime III the system is bistable and depending on the initial condition it will attain either the tolerant or the immune state. Reaching one or the other state in regime III is essentially determined by the initial fraction of Regulators per APC (R/A) in the system (i.e. higher values of this fraction led to the tolerant state while lower values lead to the immune state). This result is easy to understand qualitatively since for low values of R/A the frequency of inhibitory events, which depend on the formation of multicellular conjugates containing Effectors and Regulators on the same APC, is reduced. As proposed before (Leon et al., 2000) being able to interpret the dominant tolerance phenomenology requires that the model operates within the bistability regime. Therefore several parameters in the simulations reported here were basically set to values that guaranteed a mean clonal dynamic in the periphery operating in the regime III, around the black dot in fig 2a (Ke=Kr=0.03, A=10, Pe=1.38, μ=0.28, d=0.23, s=5). Note nevertheless that in the extended model, each individual clone operates in a different dynamical regime (according to the digram of fig 2a) given the fact that they differ in their particular value for the conjugations constants (Ke and Kr for effectors and regulators respectively). Moreover, changing the dynamic parameter μ or the parameter Km in our simulations, the set point for system dynamic can be also freely switch between different parameters regimes.

Particularly interesting to qualitatively understand the results of our simulations are the parameter dependencies in this basic model with the conjugation constants (Ke and Kr) and the dependence with the number of APC (A). Note that proportionally changing both Ke and Kr imply moving throughout the dashed line in the parameters space of fig 2a, an effect that can be also recovered by changing the concentration of APC (A). Actually increasing the conjugations constants or the APC number moves the system deeper into parameter region

TABLE 1

Primary Control Parameters

Parameter	Units	Definition	Value
A	1/Vol	Density of APCs in the periphery	----
V	Vol	Volume of the peripheral compartment	----
μ	1/days	Growth rate constant of Regulatory cell population.	----
K_m	Vol	Maximum value for the conjugation constant.	----
St	$(\text{Vol days})^{-1}$	Total number of cells entering the periphery per time unit	----
$W_{\max}$	None	Maximal value of conjugation constant allowed to escape thymic selection	----
Pr	None	Probability that a given T cells recognize a given epitope	----
P_F	None	Fraction of MHC molecules at the APCs occupied by foreign antigen	----

Primary Parameters with predefined values

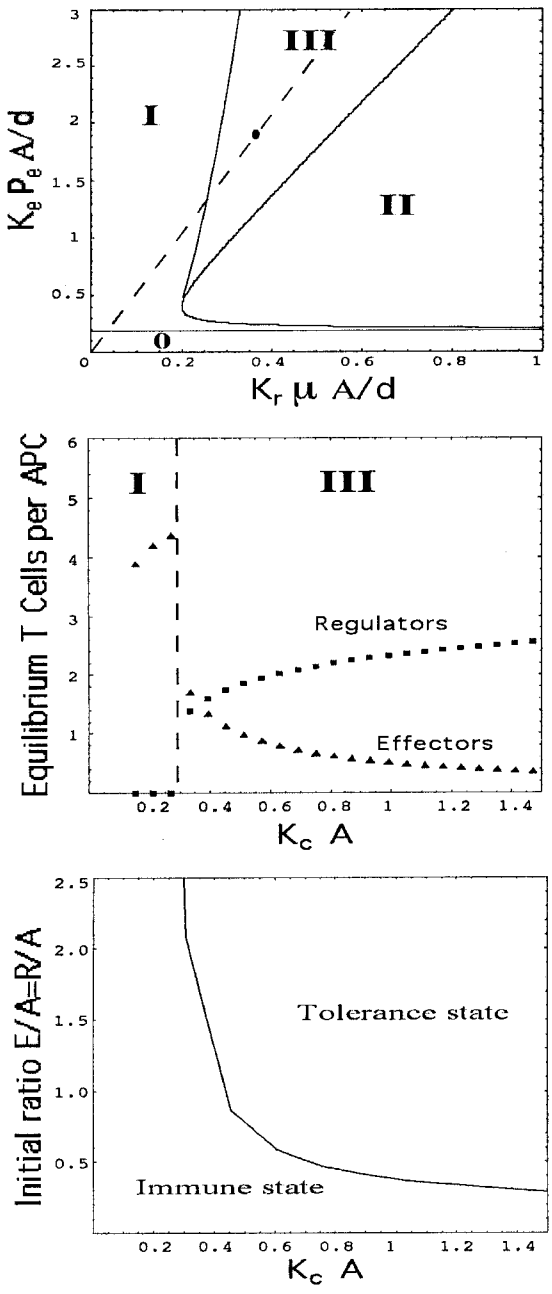
Parameter	Units	Definition	Value
S	None	Maximal number of conjugation sites per APC.	5
η	None	Switch model variable.	0
P_e	1/days	Proliferation rate constant of Effectors when stimulated by APC.	1.38
D	1/days	T cell death rate	0.23
M	1/Area	Concentration of MHC epitopes at the APC surface	1
H	None	Coefficient controlling the degree of cooperativity in the conjugation process.	Typically- 2
Sc	1/days	Number of clones entering the peripheral compartment per unit of time	8.0
α	None	Fraction of Regulators in a newly generated clone	0.5
N_S	None	Number of self-antigen epitopes	10
p_l^o	None	Fraction of self-epitopes l in the APCs before foreign antigen introduction.	In section 3.2

Secondary Parameters and intermediate quantities computed from primary parameters

Parameter	Units	Definition	Formula
p_l	None	Fraction of MHC molecules at the APCs occupied by epitope l.	$p_l = \begin{cases} (1-p_F)\, p_l^o & \text{if } 1 \le i \le N_s \\ p_F & \text{if } i = N_s + 1 \end{cases}$
C	None	Number of T cells in the new clone i entering the periphery	$= S_t / S_c$
$W_{\min}$	None	Lower limit for conjugation constants in thymic selection	$= 0.01 \cdot W_{\max}$
$k_{\min}$	Area	Lower limit for the random generation of affinities $k_{i,l}$	$= \frac{m^{-1}}{0.7} \cdot \left(\frac{W_{\max}}{1 - W_{\max}} \right)^{1/h}$
$k_{\max}$	Area	Upper limit for the random generation of affinities $k_{i,l}$	$= \frac{m^{-1}}{0.001} \cdot \left(\frac{W_{\min}}{1 - W_{\min}} \right)^{1/h}$
$k_{i,l}$	Area	Affinity of the interaction of the TCR of T cell clone i with the epitope l.	Randomly Generated $k_{\min} \le k_{i,l} \le k_{\max}$
σ_i	None	Intermediate function to compute Ki	$= m \sum_{l=1}^{L} k_{i,l}\, p_l$
Ki	Vol	Conjugation constant of the clone i with the APCs	$= K_m \frac{(\sigma_i)^h}{1 + (\sigma_i)^h}$

III, leading to: 1) An increase in the ratios R/A and R/E obtained at equilibrium in the tolerant state, as illustrated in fig 2b. And 2) An increase in the basin of attraction of the tolerant state, as illustrated fig 2c. So qualitatively, a minimal value of the conjugation constants and the APCs concentration is required in this basic model to recover tolerant states (to stay in region III), and the higher the values of these quantities the more robust and easy to reach the tolerant state would be.

We now turn to analyze our extended model where multiple populations of Effectors and Regulators are present in the system and where constant generation of new T cell clones is included.



3.1. Natural tolerance to self-antigen.

We first asked how the immune system interacts dynamically with self-antigens. To address this issue simulations were performed in which the peripheral compartment is set initially empty of T cells and is filled up by thymic output. 10 self-antigens are used in the simulation, being their relative representation on the APC given by:

$$p_l^o = \begin{cases} 0.75 & if\ l=1 \\ 0.031 & if\ 1<l<10 \\ 0.002 & if\ l=10 \end{cases}$$

There are two alternative outcomes of these simulations, which recapitulate the two alternative steady states of the basic two-population model. In one case, interpreted as the autoimmunity state, the Effectors rapidly accumulate in the periphery and out-compete the Regulators that expand only transiently (fig. 3a). In the second case, interpreted as tolerance state, after a transient expansion of Effectors, the periphery reaches a dynamic equilibrium characterized by a relatively low total density of cells, and a balanced proportion of Effectors and Regulators (fig. 3b).

In the tolerance state different self-antigens are recognized differentially in the periphery (fig 4a) both in terms of total concentration of specific T cells as in terms of the balance of specific Effectors and Regulators. Antigen l=1, which is over represented in the system, causes deletion of specific precursor T cells in thymus and consequently it is not recognized at the periphery. Antigens l=2-9 which are represented in the system at an

Fig.2- Dynamical properties of the basic model. **A-** Different parameter regions in the model, depicted in the plane ($K_e P_e A/d$) vs ($K_r \mu A/d$) with s=5. There are 4 parameter regions in the model. Regions 0, I, II result in globally stable regimes, where the only stable equilibrium are respectively the trivial state (E=0, R=0), the immune state (E>0, R=0) and the tolerant state (E>0 and R>0). The parameter region III corresponds to a bistable regime where both immune and tolerant states are stable, and could be attained depending on the initial proportion of E and R cells. **B-** Changes in the equilibrium concentration of regulators and effectors cells in the tolerant state, when simultaneously changing the conjugation constants for the effectors and regulators or the total number of APC (A). Increasing these quantities increment the proportion of regulators at the tolerant equilibrium, making it more robust and difficult to break. **C-** Minimal values of the initial fraction of Effectors and Regulators per APC, which leads to the tolerant equilibrium, as a function of the conjugation constants K=Ke=Kr and the number of APC A. Increasing these quantities enlarges the basin of attraction for the tolerant state, since it can be attained from lower initial value for the ratio of regulators per APC.

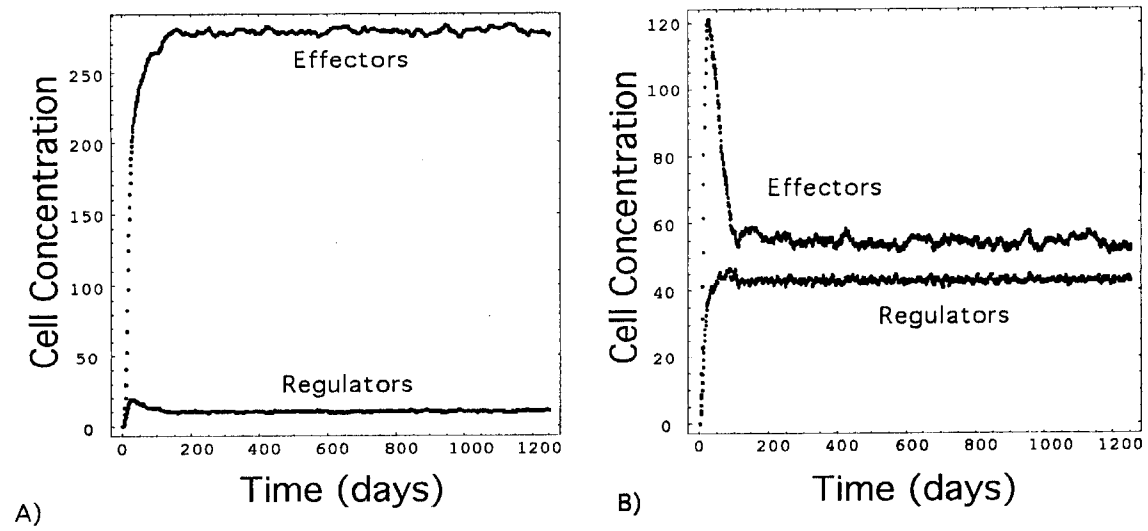


Fig.3- Dynamical evolution of the model in the interaction with self-antigens. Two alternative equilibriums are obtained in simulations. **A-** Autoimmune state. **B-**Self-tolerant state.

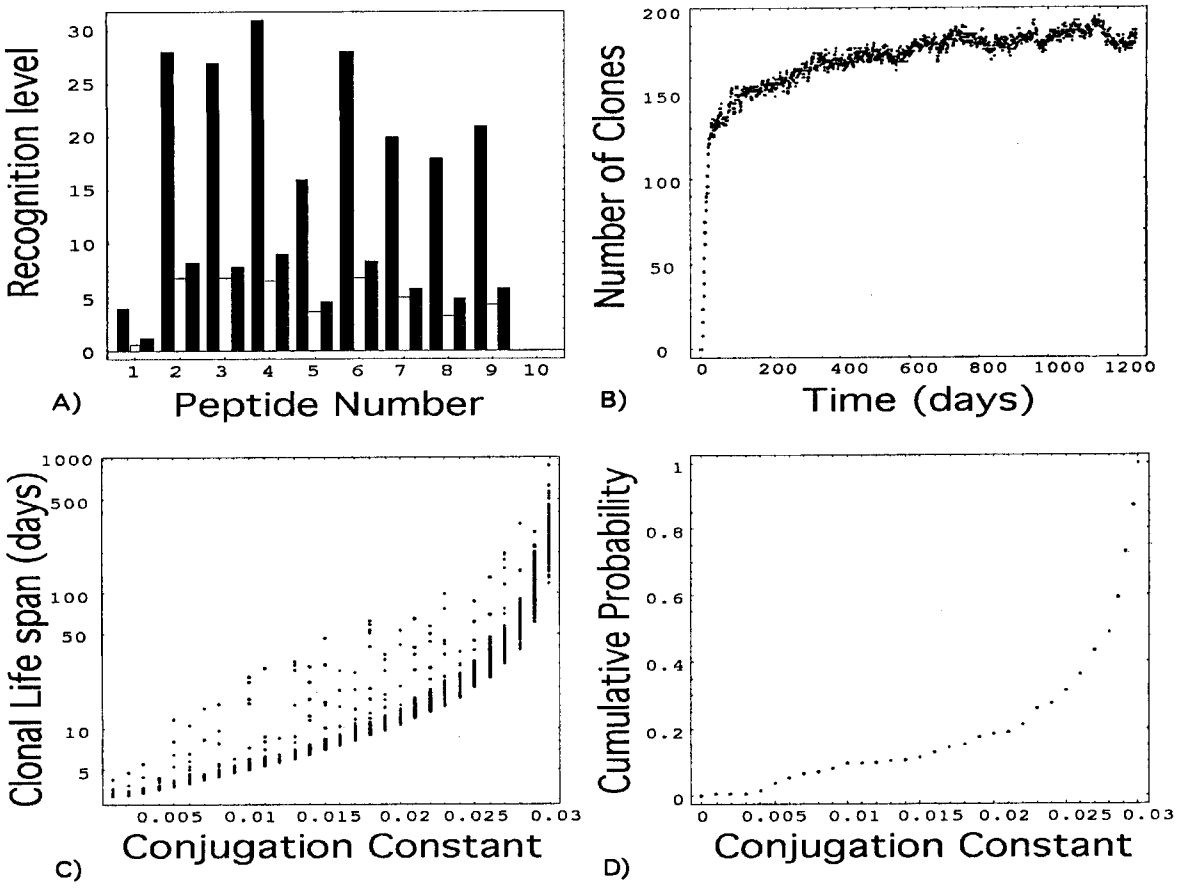


Fig .4- Properties of self-tolerance state. **A-** Differential recognitions of self-peptides in the tolerant equilibrium. For each antigen epitope (given by its index) the number of clones (solid bars),concentration of effector cells (empty bars) and concentration of regulatory cells (dashed bars), which recognize it, are shown. **B-** Gradual increase in the number of clones in the niche, while the model attains the tolerant state. **C-** Direct correlation between the life span of individual clones and its conjugation constants to the APC in the niche. Each point in the graph represent an individual clone in a 3 years long simulation. **D-** Preponderance of clone with high conjugation constants in the tolerant state. The cumulative distribution of clones according to the values of their conjugation constants in a late time point of a tolerant simulation is shown..

intermediate level, are recognized by both Effectors and Regulators in the system. Antigen I=10, which is poorly represented in the system, is consequently poorly recognized by both Effectors and Regulators. The Regulator populations represent typically between 30% and 95% of the total T cells in the niche (around 50% in the case shown in fig 3b), being the particular value strongly dependent on particular parameters choice. As to the diversity of the peripheral repertoire, the number of peripheral T cells clones slowly increases up to a maximal value characteristic of tolerance state (fig 4b). At equilibrium, T cells clones constantly turn over with life spans that range from 3 day to several months (fig 4c). The clonal life span is directly correlated with the conjugation constant. The peripheral T cell repertoire is biased towards high conjugation constants as shown in the cumulative distribution in fig 4d. Another important result is that there is a smooth distribution in term of clonal sizes in the periphery (data not shown), indicating that no single clone dominates the tolerant state.

To study dependence on parameters values we performed 100 simulations for each parameter set, computing the frequency of different dynamical states obtained. Increasing St decreases the frequency of autoimmune states obtained in simulations (fig.5a). This result is quite intuitive since increasing St leads to a direct increase in the number of Regulators entering the periphery per unit of time and so it contributes to increase the ratio R/A thus favoring the establishment of tolerance. The dependencies on other parameters can be summarized in a single diagram (fig 5b) where the axes are particular parameters combinations and the lines represent parameter sets that will lead to tolerance in 50% of the simulations for the indicated value of μ. Moving in this parameter space below the line increase the incidence of autoimmunity and moving above the line increase the incidence of tolerance. For instance the previously shown dependence with the size of thymic output St is easily recovered in this diagram since increasing St implies to move upwards along vertical axes, so that we go from low to high frequency of tolerant states. But a more complex and interesting dependency on the parameters controlling thymus output becomes evident in this representation. Note that changing parameter St, which controls the magnitude of thymic output, moves the system along the y axis while changing parameter Wmax, which controls the "quality" of self-antigen recognition in the thymic output, moves the system along the x axis. So the results in fig-5b show that allowing a higher conjugation constant to escape thymic selection can compensate a decrease in the absolute size of thymic output, keeping the capacity of the model to tolerate self-antigens. In other words, to explain tolerance to self–antigens, thymic output must be adjusted so that for a particular value of St, clones with self-antigen recognition higher than a given value are allowed to appear at the periphery. This result of the simulations can be qualitatively understood from the properties described in section 3.1 for the basic model. Note that increasing the conjugation constant for Effectors and Regulators in the periphery is expected to favor the tolerance state, so that it can now be reached even for initially lower values of the ratio R/A that relate in the simulations to lower values of St.

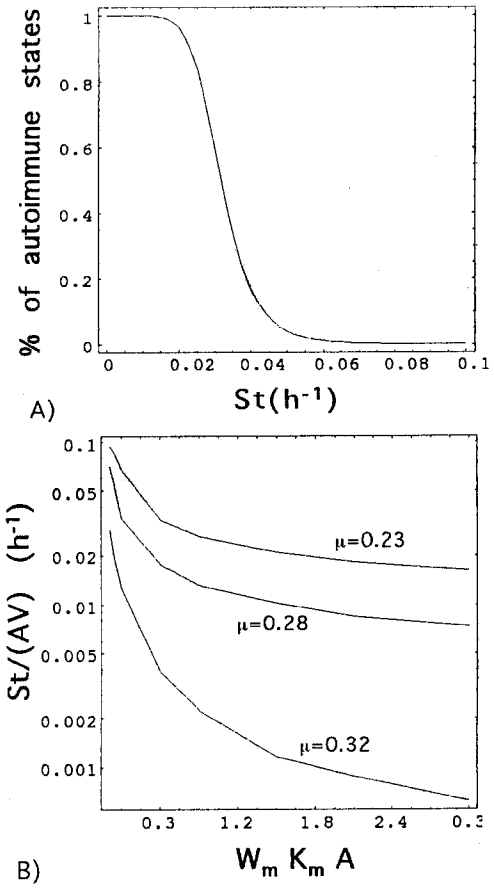


Fig .5- Parameter dependencies of the incidence of tolerant and autoimmune states recovered in the simulations. **A-** Decrease in the incidence of autoimmune states with the total number of T cells exported per unit of time to the niche from the thymus (St). **B-** Parameter chart for the dependencies of the incidence of tolerant and autoimmune states. The axes in the graph are complex combination of parameters in the model. The lines represents the points in this space where 50% incidence of tolerant states is obtained for the indicated value of μ. For each μ, moving below the line increase the incidence of autoimmunity and moving above the line increase the incidence of tolerance.

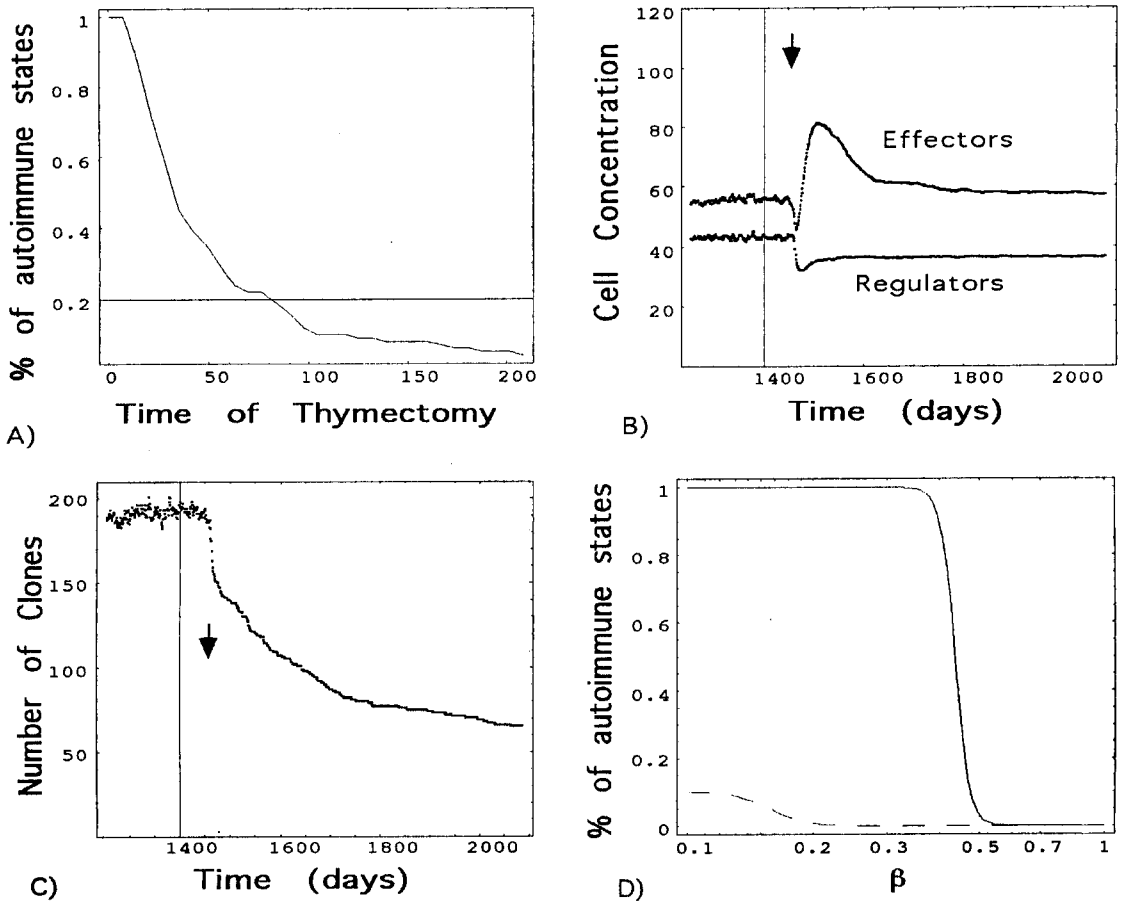


Fig .6- The role of Thymus in the tolerance to self-antigens. **A-** Dependence of the incidence of autoimmune states with the time to perform thymectomy in the model. The thymus is relevant in the initial onset of the tolerant state. **B-** Dynamic evolution of the model upon thymectomy in an already establish tolerant state. The arrow indicates the time point of thymectomy in the simulation. After a small transient the concentrations of E and R cells in the niche remain close to the original values. **C-** Dynamic evolution of the number of clones in the niches upon thymectomy in an already establish tolerant state. Diversity is drastically reduced due to inter-clonal competition. **D-** Incidence of autoimmune states induced in the model, by an immuno-suppression treatment that instantaneously reduces to a fraction β the initial concentration of every existent T cells clone in an established tolerant state. Solid and dashed lines are respectively for a system where thymectomy has or has not been performed previously to the suppressive treatment. The continuous thymic output positively contributes to the stability of the existent tolerant state.

In summary, the results of this section show that the simulated immune system has a dynamical equilibrium, where the balance between Effectors and Regulators in the periphery can be interpreted as a state of active tolerance to self-antigens. The parameter conditions to reach such a tolerant state relate to the parameters that control thymus output, both in terms of its magnitude and in terms of the strength of self-recognition allowed by thymic selection.

3.2. The role of Thymus in Natural tolerance.

Given the results of previous section, we asked whether or not a constant source of new T cells clones is required to guarantee tolerance during the whole simulation. To address this issue the simulations of the previous section were reproduced, but now the thymic output St was set to zero at a given instant Tt, mimicking a thymectomy (fig-6). The result shows that source of new cells is only required to promote the tolerant state at the beginning of the simulations, and is not required to ensure the persistence of the tolerance state (fig. 6a). Nevertheless, thymectomy does have a strong effect even it is performed later (fig 6b and c). The total number of T cells and the balance Effectors versus Regulators is only marginally affected by thymectomy (fig. 6b), but the diversity in the T cell repertoire is drastically reduced upon thymectomy (fig.6c). Actually, in long enough simulations only the highest conjugation constant clone will remain in the periphery. The latter simulation result is just an expression of the competitive interaction among different T cells

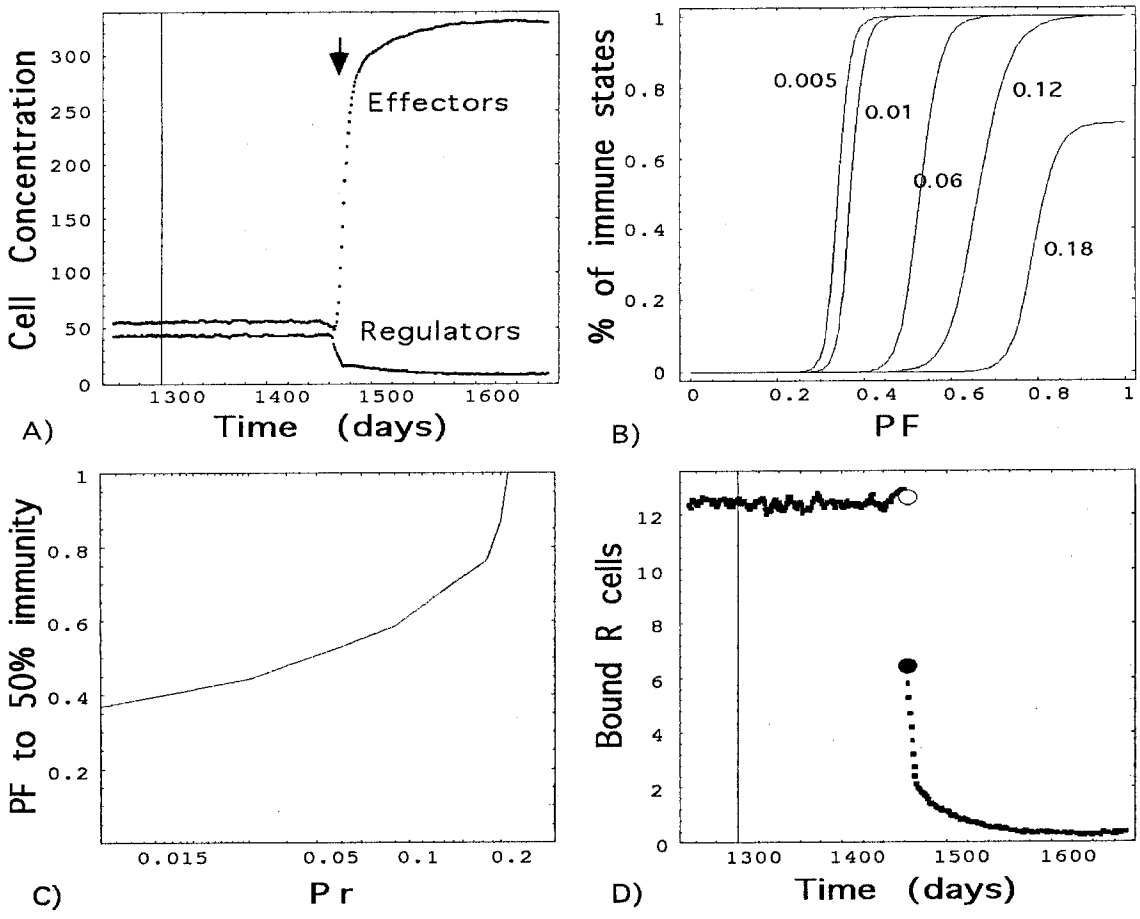


Fig .7- Response of the model to the introduction of a foreign antigen. **A-** Typical dynamic evolution of the model in an immune reaction. The arrow indicates the time point for the introduction of the foreign antigen. **B-** Dependency of the incidence of immune states recovered in the simulation with the relative representation of the introduced foreign antigen in the surface of the APCs in the niche (PF) for different values of the recognition probability in the system (Pr explicitly indicated value in the graph). **C-** The same dependence that in **B**, but represented as the dependence of the value of PF to obtain 50% incidence of immune state (measurement of the system reactivity) with the recognition probability Pr. **D-** Dynamic evolution of the concentration of R cells bound to the APC in the course of immune reaction. Upon the foreign antigen introduction a net displacement of the regulatory cells for the APC surface is observed.

clones, which operates in the periphery and plays a critical role. In appendix A we show analytically that competition is expected from the peripheral dynamic in this model both in the immune and in the tolerant equilibria.

Another role of thymic output in the simulations is to stabilize the tolerant state, avoiding reversion to the autoimmune state after a given perturbation. This idea is illustrated in a simulation where an tolerant system is perturbed by instantaneously reducing to a fraction β the size of all the clones. This perturbation simulates, in the simplest way, a typical immune suppression treatment, for instance through irradiation or some cytostatic drug administration. When this perturbation is made in the absence of thymic output the incidence of autoimmunity increases as β decreases (fig.6D-solid line). In the presence of thymic output the tolerance state is comparably more resistant to the perturbation.

Summarizing, this section shows that thymic output is essential to organize the tolerance to self-antigen but it is not critical to maintain it later on. Nevertheless, the continuous presence of the thymus makes the tolerance state more robust and is necessary to maintain peripheral T cell diversity.

3.3. Immune reaction to foreign antigens.

Having understood how the simulated immune system couples tolerantly with self-antigens, we asked how it reacts to the introduction of a *foreign* antigen. To address this issue we made simulations, in which a tolerance state (obtained as described in section 3.2), is perturbed by the introduction of a single *foreign* antigen, whose fractional representation in peripheral APCs raises instantaneously from zero to P_F. Two types of results were obtained. The introduction of the foreign antigen may barely affect the balance of Regulators and Effectors in the periphery,

which remains in the tolerance state (results not shown). Alternatively the introduction of the foreign antigen may drastically subvert the tolerance state (fig 7a). In these situations, which we interpret as immune response to the foreign antigen, the Effectors expand very fast and out compete the Regulators in the periphery. The frequency of tolerance or immunity in these simulation depends primarily on the relative representation of the *foreign* antigen at the APC surface (PF) and the probability that the new antigen is recognized by an arbitrary T cell clone (recognition probability Pr) (fig 7 b and c). The frequency of immune responses increases with the increase of PF for different values of the recognition probability Pr (fig.7b). Also the value of P_F required to get immune responses in 50% of the simulation increases for higher values of Pr (fig.7c). In practice, immune responses are only reliably obtained in the simulations using high values of P_F and low values of Pr.

These properties of the simulation are easy to understand qualitatively if one considers the dual effects of the introduction of the foreign. On the one hand, the APC conjugation constant for the fraction (Pr) of the T cell clones that specifically recognize the new antigen is increased (those clones with $k_{i,Ns+1}$ different from of zero). On the other hand, the antigenic presentation of the self-antigens decreases linearly with P_F (eqn. 2), and the APC conjugation constant for the remaining T cells clones in periphery is reduced accordingly (eqn. 3). The behavior of the model can be finally understood by following the number of peripheral Regulators that effectively interact with the APC. Systematically, in simulations that result in immune responses the total number of Regulators conjugated with the APC drops significantly after the introduction of the foreign antigen (fig 7d). This drop in Regulators requires two conditions: 1) that just an small fraction of the total T cell clones recognize the new antigen (low values of Pr); and 2) that the conjugation constants of T cells direct against the self-antigens are considerably reduced (i.e. high values of PF).

Several parameters can be tuned in the model to increase probability that there is an immune response to foreign-antigens in the simulations. First, immune responses are facilitated by degree of cooperativity in T cell-APC conjugation (fig 8a). The higher the value of parameter h (defining the cooperativity) the lower the value of P_F required to obtain immune states in 50% of the simulations. Qualitatively increasing h increases the sensitivity of T cells conjugation constants to changes in the concentration of the epitopes on the APC . For high

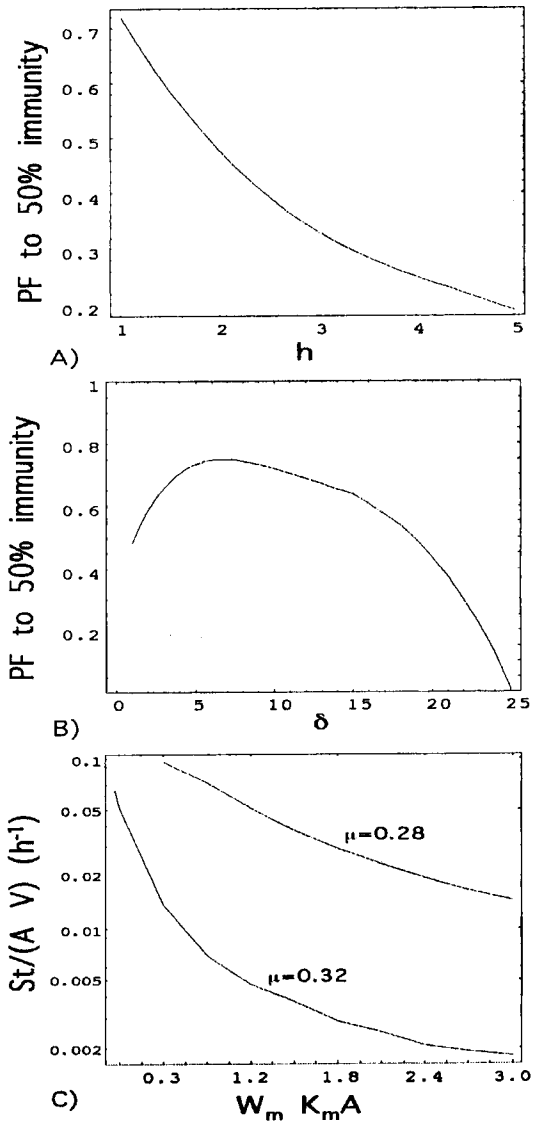


Fig .8- Other parameters dependencies of the incidence of immune responses to foreign antigens in the model. **A-** The value of PF to obtain 50% incidence of immune states decrease with the increase in parameter h that control the cooperativity in the process of APC-T cells conjugation. **B-** The value of PF to obtain 50% incidence of immune state first increase and then decrease with the increases in parameter δ. This parameter gives the factor by which the number of APC in the niche has been instantaneously increased upon the foreign antigen introduction to simulate the activity of the innate immune system (see text). **C-** Dependency of system reactivity with the pre-existent tolerant state. The axes in the graph are complex combination of parameters in the model that control the organization of the lately perturbed tolerant state. The lines represents the points in this space where 50% incidence of tolerant states is obtained upon foreign antigen introduction for the indicated value of μ. For each μ, moving below the line increase the incidence of immune states and moving above the line increase the incidence of immune states.

values of h a very small displacement of self-antigens by foreign antigen, may lead to large changes in the value of the conjugation constant.

Second, the immune responses can be favored because the introduction of a foreign antigen is always concomitant with additional perturbations (mediated by the innate immune system) that synergize with the changes in the fractional representation of epitopes. An example of such perturbations is the increase in the number of APCs in the periphery (Leon et al., 2000). We performed simulations in which the introduction of foreign antigen was concomitant with an increase by factor δ of the number of APCs. The value P_F necessary to obtain immune responses in 50% of the cases first increases and then decreases as a function of δ (fig. 8b). In practice if the value of δ is large enough an immune response (or more precisely autoimmunity) can be triggered even without introducing a new antigen (P_F=0). This result stems from two effects of the increase of the number of peripheral APC (A) on the dynamics. On the one hand, increasing A reinforces the tolerance state making it more probable. On the other hand, increasing A, directly reduces the initial ratio R/A in the periphery and therefore promotes the immune response state. The interplay of these two opposite tendencies explains the curve in fig 8b. For relatively low value of δ the first effect predominates favoring tolerance, while for sufficiently large values of δ the latter effect become dominant favoring immune responses.

Finally, immune responses may be favored by the parameters that make the tolerant state with self-antigens less robust. Simulations were performed where the system was allowed to attain tolerance with the self-antigen and a foreign antigen introduced fixing the parameters defining the foreign antigen perturbation (P_F=0.8, P_r=0.02 and δ=0) and systematically changing the remaining parameters. The results are summarized in the parameter chart in fig 8c. The axes in this graph are complex combination of simulation parameters, and the lines represent parameter sets that result in 50% incidence of immune states. Each line represents a particular value of μ. Moving in this parameter space bellow the line increases the incidence of immune reactions, while moving above the line decreases the incidence of immune reactions. Immune responses are disfavored by increasing the magnitude of the thymic output (St) and/or by increasing the values of conjugation constants allowed at periphery after thymic selection (Wmax). These properties of the simulation can be qualitative understood from the properties of the basic model (section 3.1). On the one hand, increasing St directly increases the number of Regulators per APC in the periphery, thus favoring tolerance to foreign antigen. On the other hand, increasing the value of conjugation constants for Effectors and Regulators reinforces the stability of the tolerant state, which becomes more robust to the perturbation by the introduction of the foreign antigen.

In summary, this section shows that the simulated immune system can react to the introduction of a foreign antigen, switching from a tolerance state to an equilibrium state that can be interpreted as an immune reaction. Qualitatively the model reacts to the change on the relative representation of antigens at the APC surface, which affects the conjugation constant of different T cells clones in the periphery, significantly displacing part of the Regulators from the APC.

3.4. Autoimmune reactions to change in representation of self-antigens.

The results of the previous section lead naturally to a question on how the system would react to perturbation to the representation of the self-antigen themselves. To address this question, simulations were performed similar to the ones of section 3.4 but in which the fractional representation at the APC (Pl) of the self-antigen (l) was instantaneously increased displacing the remaining self-antigens. The results of these simulations depend of the self-antigen, which is perturbed, particularly on the number of Effectors and Regulators that specifically recognize it in the preexistent tolerance state. For the self-antigen l=1, there is no way to get an immune reaction, since there are no T cell clones specific in the periphery (see fig 3). For self-antigens l=2-9, which are strongly recognized by peripheral Effectors and Regulators (fig 3) it is rather difficult to get immune reactions. The most frequent scenario is a transient expansion of specific Effectors that is rapidly controlled by a subsequent expansion of specific Regulators (fig. 9). Interestingly, for these self-antigens, once you have induced this transient reaction and the original self-antigen representation is restored, the system becomes transiently refractory to a new perturbation, i.e. raising the concentration of the same self-antigen may fail to cause the same type of transient immune reaction (fig 9). Finally for self-antigen l=10, which is poorly recognized in the periphery (fig 3), the system can easily switch to the (auto) immune state (not shown), being the overall behavior regarding this antigen similar to that of a *foreign* antigen.

Qualitatively the results in this section and the previous one show that self-non self discrimination in this model is

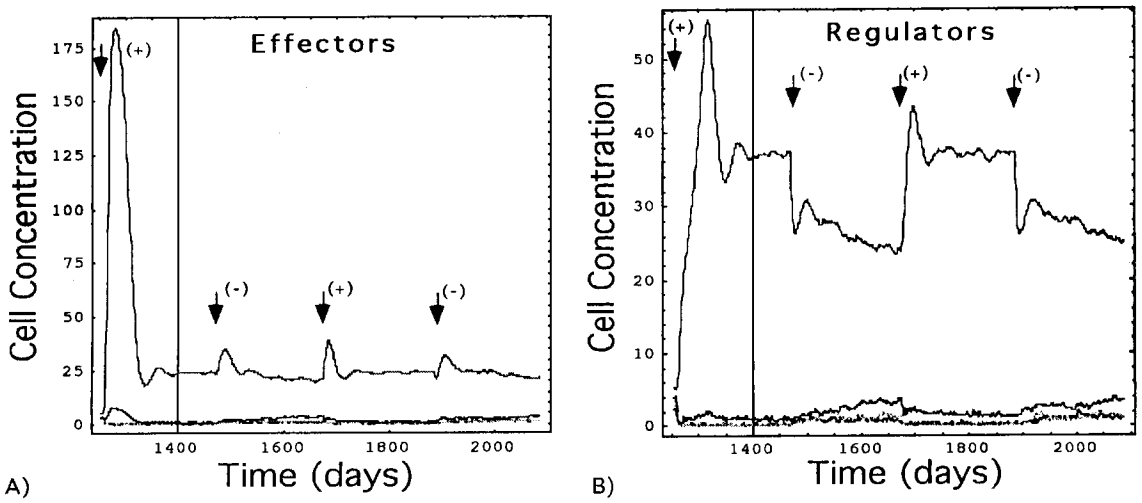


Fig.9- Response of the model to the abrupt variation of the concentration of a given self-antigen. The dynamical evolution of the concentration of effectors **(A)** and regulator **(B)** is depicted. Each line in the graph corresponds to the cells that recognize a given self-antigen. The upper line is the response to the self-antigen whose concentration is varied in the simulation. Arrows plus a + or - symbol indicate time point where the self-antigen concentration has been increased or decreased respectively. The result shows a transient expansion of the specific effectors that is rapidly followed by the expansion of specific regulators, upon the increase in the self-antigen concentration. But after reducing back the concentration of the self-antigen the concentration of specific regulators is kept relatively high in respect to the concentration of regulators for other self-reactivity's. Raising again the self-antigens concentration does not produce the same transient expansion of the specific E cells in the system.

just based in a quantitative difference of the frequency of recognition by T cells in the periphery. Self-antigen appears tolerant, except in the extreme case of repertoire hole (m=1), when the number of T cells that recognize them specifically is high. Foreign antigen or very poorly represented self-antigens trigger immune responses in the simulations, since they are recognized by a small fraction of T cells in general and Regulators in particular. Two major aspects contribute in the model to ensure a high frequency of self-antigen reactivity: first, positive selection on the thymus enriches the output of new T cells with self-antigen reactive cells; and second, clonal survival in the periphery increases with the conjugation constant to self-antigens further enriching the peripheral pool of self-antigen specific T cells.

3.5. Coexistence of natural tolerance and Immune activity.

We studied separately the parameter conditions that favored the establishment of tolerance to self-antigen epitopes (section 3.2) and reliable immune responses to the introduction of a foreign antigen (section 3.4). In this section, we ask what conditions allow for these two behaviors simultaneously. The result of this analysis is shown in fig 10, where results previously shown in fig 5b section 3.1 (solid lines) and in fig 8c section 3.3 (dashed lines) are brought together for the value μ=0.28. Three regions (denoted A, B and C) can be defined in this complex parametric space. In region A the probability of organizing a tolerant state to self-antigen is low, although the capacity to make an immune response to a foreign antigen is high. In region B the probability to tolerate self-antigens is high, but the immune response to foreign antigens is disfavored. Finally, in region C there is a compromise and the system is systematically tolerant to self-antigens and responds reliably to foreign. The area of this region C decreases as the value of μ increases (not shown).

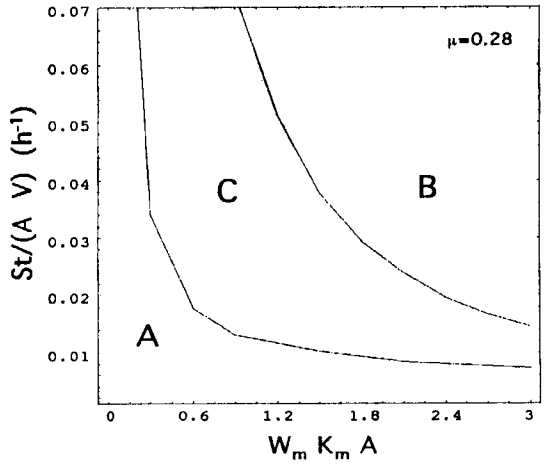


Fig.10- Parameter condition for the coexistent of tolerance to self-antigens and immunity to foreign antigen. The graph is the composition of fig 5b and fig 8c μ =0.28. It delineates three parametric regions in the overall model behaviour (see text).

Region C corresponds to the most realistic parameter settings in the simulations. In order to be in region C several conditions on the parameters must be met. The magnitude of thymic output (St) must be above a lower threshold. For a given value of St, the parameter Wmax, which controls the process of thymic selection, has to be within an interval. Qualitatively the model requires a minimal thymic output and both positive and negative selection processes. It requires positive selection to ensure the generation of enough Regulators recognizing self-antigens, with sufficient strength as to reliably organizes a tolerance state. It requires negative selection of clones with too high conjugation constant to APCs presenting self-antigen so that the tolerant state established is not too robust making, otherwise it would be impossible to break it by the introduction of a foreign antigen.

3.6. Reestablishment of natural tolerance after an effective immune response.

The natural outcome of mounting an immune response is the elimination of the foreign antigen and the restoration of self-antigen presentation. The question we turn our attention to in this section is: what happens after the foreign antigen is cleared? To study this issue, simulations were performed, which were initially in the immune equilibrium (similar to the ones produced by protocols described in section 3.3) and where the foreign antigen is eliminated by setting P_F to zero, restoring instantaneously the preexistent levels of self-antigen presentation. Two possible outcomes are observed in this experiment. The system reverts to the tolerance state controlled by Regulators (fig11a) or the system remains dominated by Effectors being this final state interpreted as autoimmunity (fig 11b). The parameter conditions that facilitate the reestablishment of tolerance state, are related to the thymic output (results not shown) The higher the values of thymic output (St) and of self-antigen dependent conjugation in the thymus (Wmax), the higher the probability that the peripheral populations revert to the tolerant equilibrium. Nevertheless, these conditions are not very stringent. It is relatively easy to observe reversion to tolerance under those parameter settings, which allowed the system to respond to foreign antigens (namely inside parameters region C in fig 10). Only in a relatively thin layer of region C, close to the lower limiting curve, the capacity of the model to reestablish tolerance after the immune response is lost (data not shown). Qualitatively, the role of thymic output in the reestablishment of tolerance is easy to understand. Note that during the immune reaction, peripheral Regulator populations are markedly reduced. Therefore, the only way to restore a tolerant state is to provide a sufficiently large influx of new Regulators (large St). The higher the conjugation strength of these Regulators to the APCs the higher their effectiveness.

4. Discussion.

This article presents a simulation of the immune system involving the thymic generation and peripheral dynamics of many Regulators and Effectors cell clones. This simulated immune system is able to establish tolerance to ubiquitous and constitutive self-antigens and reliably mount effective immune responses upon the introduction of other (foreign) antigens.

In our simulations a pool of Regulators that are driven by constitutive self-antigens ensures peripheral tolerance. These Regulator clones establish a dynamical equilibrium with Effector clones, where the total size of the two pools is mutually controlled. The steady state obtained in the presence of Regulators is characterized by lower numbers of total cells than would be found in their absence. Qualitatively, this result is readily consistent with the observations that CD4 T cell expansion and recovery in adoptive transfers depends on the presence or absence of regulatory CD4+CD25+ T cells (Annacker et al., 2001b). The proportions of Regulators and Effectors in the periphery could potentially offer another way of assessing the realism of the simulations. This is complicated by the fact that the Regulator and Effector cells cannot be identified as such in the real immune system Most regulatory activity seems to be contained within the CD4+CD25+ T cell population which represents 5-10% of all CD4 cells. Thus the real proportion of Regulators is expected to be around this value. In simulations tolerance is only reliably obtained under parameter values that result in steady states where the fraction of Regulators is never smaller than 30% and very often reaches 50 to 90%. The manifest inability of the model to show tolerance simultaneously with a small fraction of Regulators stems from the mechanism of suppression in multicellular conjugates. Numerical analysis of the basic two T cell populations model (Leon et al JTB) shows that smaller fractions of Regulators can be obtained if APC can conjugate simultaneously with more than 50 T cells or if the conjugation constant for Regulators is much higher than that of the Effectors. The first conditions is probably unrealistic for steric reasons even considering that dendritic cells may have a large surface to volume ratio. The second condition is more reasonable having been postulated before (Modigliani et al., 1996) and shown recently (Jordan et al., 2000; Jordan et al., 2001) that high

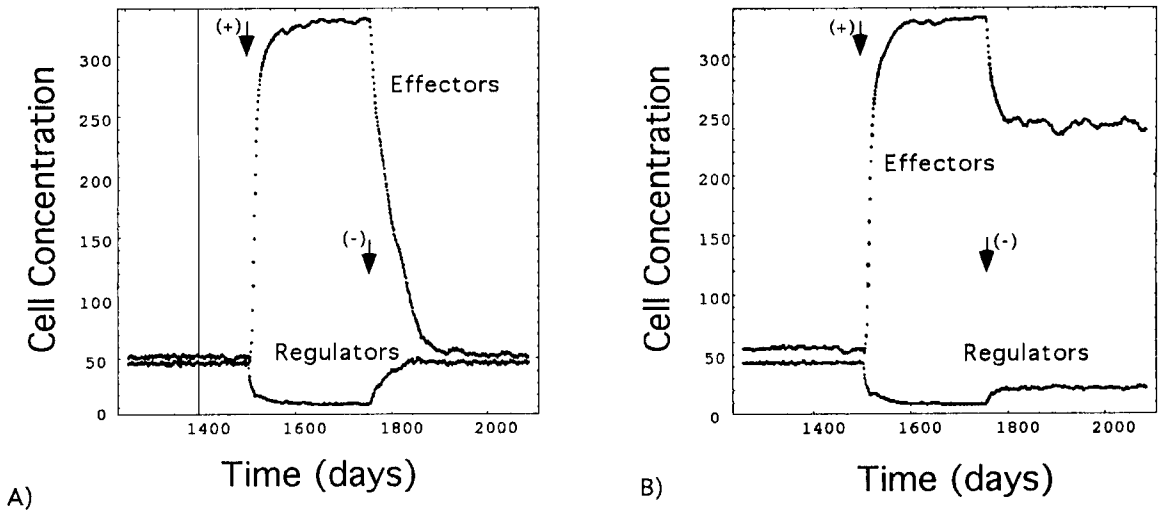


Fig.11- Characteristic dynamical evolution of the model after eliminating the foreign antigens in the course of immune reaction. Arrows plus a + or - symbol indicate time point where the foreign antigen has been introduced or eliminated respectively. **A-** Reestablishment of tolerance in the peripheral compartment after the immune reaction. **B-** Autoimmunity following antigen elimination

affinity interactions may educate thymocytes to acquire Regulator phenotype. This possibility suggests a straightforward extension to our simulations that assumed that Regulator generation is antigen non-specific. Alternatively, we can consider that the pool of CD25- T cells that represents 90-95% of the CD4 population is heterogeneous containing both Effectors and cells that are not stimulated by the self-antigens. According to our simulations the fraction of Effectors and Regulators should be comparable. This is compatible with an interpretation that only 5-10% CD4+ T cells are Effectors whose proliferation is driven by self-antigens and the remaining cells are unable to driven proliferate by these self-antigens. The suggestion is that the activated/memory compartment of CD45Rblow in normal mice, containing comparable numbers of CD25+ and CD25- (respectively Regulators and Effectors), corresponds to those cells driven by self-antigens described by our model. Within this line of interpretation, our model does not account for the persistence and homeostatic proliferation of naïve CD45Rbhigh T cells.

Clonal diversity is the hallmark of the immune system. T cell diversity is generated in the thymus and dynamically maintained in the periphery (McMahan & Fink, 2000). Our model is limited in the capacity to account for maintenance of peripheral diversity. We have assumed that peripheral populations are competing for conjugation with the APCs. De Boer and Perelson (De Boer & Perelson, 1997)have pointed out that under these conditions, clonal diversity is limited by the heterogeneity of the APCs: each subpopulation of APCs will drive a single clone that excludes others by competitive exclusion. Our model presents an improvement to this scenario. Parameters can be set such that at the tolerant steady state the APC conjugation sites are not saturated, which reduces the stringency of competition but does not prevent competitive exclusion. Under these conditions the new clones produced by thymus persists transiently but diversity is lost if thymic export stops. Given this property of the model it is tempting to interpret the progressive loss of diversity in peripheral pools with aging as the result of thymic involution (Mackall & Gress, 1997). However, the loss of diversity following adult thymectomy as predicted by our model follows a fast kinetics that is unrealistic. With a turnover of 4% of cells per day a 2/3 reduction of clonal diversity in Regulators and Effectors would be practically reached within 600 days. This is an area in which our simple model needs to be extended by including antigenic heterogeneity of APCs and spatial distribution of interacting cells. These features are well known to allow for diversity in ecoevolutionary models.

There is ample evidence that the thymus is fundamental for tolerance induction in general and for the establishment and robustness of peripheral tolerance in particular. Sakaguchi et al (1985) have shown that neonatal thymectomy, but not thymectomy in the adult, may result in the emergence of multiple organs specific autoimmune diseases in mice. Moreover, adult thymectomy markedly increases the susceptibility of animals to immunossuppression-dependent autoimmunity, indicating the importance of thymic export throughout life (Sakaguchi et al., 1994; Heath et al., 1996). Our model,

where thymic selection and output are key actors, provides a straightforward interpretation for these findings, whose mechanistic base is still unclear. Reliable organization of tolerance requires the thymus to shape the emergent repertoire of Regulators and Effectors seeding the periphery with an appropriate number of Regulators per APC. Also, the return to a tolerant state after a transient loss of Regulators during an immune response requires a significant influx of Regulators from the thymus. In contrast with other theoretical proposals (Modigliani etc.) we obtained these dynamic properties without making additional postulates about the existence of a particular developmental program for the generation of regulatory cells or their antigen-dependent differentiation. This shows that antigen nonspecific differentiation of Regulators may be sufficient to explain the role of the thymus in promoting peripheral tolerance, provided that other conditions are met. Hence, reliable tolerance in our model requires that the repertoires of both Regulators and Effectors cells emerging from the thymus are strongly selected to favor interactions with ubiquitous/constitutive self-antigens. The affinity for the self-antigens must be sufficiently high as to ensure that the emergent repertoire is biased towards the self-antigens. Too high avidity implies that the self-marker specific tolerance will be two strong and therefore may prevent most responses to foreign antigens. In conclusion, our results indicate that positive and negative selection are both necessary for appropriate immune behavior. However, contrary to the dominant immunological interpretations, we suggest that positive selection would be required to ensure the generation of sufficient self-marker specific Regulators, and negative selection would be required to prevent that too efficient Regulators seed the periphery and prevent immunity to invading pathogens. Finally, It is worst to note that the model is consistent on quantitative terms to the estimated contribution of thymic output to the peripheral T cell pool. Measurements indicate that the number of thymic immigrants that enter the periphery daily represent about 1% of the peripheral pool, and according to the reference parameters in our model the fractional input of total Regulators and Effectors is 4% per day. Moreover this value can be further reduced in our simulations by raising the threshold for thymic deletion, which will results in the production of more efficient Regulators with higher conjugation constants for APCs expressing self-antigens. Therefore the non-linear dynamic of our model successfully explain how such a small thymic output of T cells could play such important role for the overall behavior of the system.

In our model tolerance to body components requires the expression of the same self-antigens both in the thymus and in the periphery. This property seems to contrast with the experimental fact that tolerance can be acquired to extraneous grafted body components. This contrast may be only apparent. Tolerance to peripheral antigens can be induced in animals by adoptive transfers of specific regulatory cells from tolerant donors. The results of our simulations are readily compatible with these findings if we assume that donor tolerogenic cells were previously shaped by thymic and peripheral selection in tolerant animals. Tolerance to peripheral antigens can also be induced by protocols where the preselection of regulatory cells cannot be so easily evoked. One such type of experiments is the classical Medawar neonatal tolerance induction to allografts. Neonatal tolerance is only established if and only if the recipients are also grafted with donor hematopoietic (Medawar, 1958) or thymic (Le Douarin et al., 1996) tissues. Under the light of our model, the requirement for hematopoietic or thymic tissues can be readily interpreted as a means of ensuring that alloantigens are presented both by the graft and by the APCs mediating thymic selection, respectively hematopoietic derived cells or thymic epithelial cells. Interestingly, this interpretation is only tenable under the light of our simulation results if epithelial cells as well as hematopoietic APCs are able to mediate both positive and negative selection in the thymus. Finally, some tolerance induction protocols are outside the scope of our model because the experimental control variables are distinct from the variables or parameters in our model. A good example is Waldmann protocol of tolerance induction by anti-T lymphocyte antibody treatment (Waldmann & Cobbold, 1998), whose interpretation according to our model would require independent but crucial assumptions on the putative effects of these antibodies in the generation of regulatory cells.

Self-tolerance mediated by linked suppression poses some hard problems that our work identifies clearly. Mounting an immune response against foreign antigens requires the system to fulfill two conditions. First the Regulators capable of recognizing the foreign antigen must be bellow a critical number relative to the number of APCs; second, the Regulators driven by ubiquitous self-antigens presented by all APC should be prevented to suppress specific Effectors. The first requirement is easily met in our simulation, by setting the parameters as to increase the specificity of the T cells, and to increase the stringency of selection that bias thymocyte repertoire towards self-antigens. The second requirement is more

difficult to achieve since sufficient Regulators against ubiquitous self-antigens is precisely what grants the reliability of self-tolerance. An immune response dominated by Effectors may be easily obtained by two non-mutually exclusive processes triggered by foreign antigen: increase in the number of APCs and loss of self-antigens on the APCs, which may synergize to prevent linked suppression by Regulators.

Professional APCs and their precursors have receptors specific for pathogen markers. The introduction of the foreign antigen often triggers via these receptors an increase in the number of APCs. As we have shown before (Leon et al., 2000), a sudden increase in the number of APCs not followed by an equally fast increase in T cells reduces the probability that Effectors and Regulators meet on the same APC. This has two consequences that synergize to facilitate an immune response dominated by Effectors: on the one hand, Effectors are less suppressed, and on the other hand, Regulators grow less because they get less growth factors.

If the foreign antigen does not lead to a concomitant increase in the number of APCs, mounting an immune response involves, as an absolute prerequisite, the displacement of the self-antigens by foreign antigenic peptides in all or some peripheral APCs. Typically, the foreign antigen must reach up to 20-30% of all the MHCs presented by the APC. Although these estimations are perhaps limited by the simplistic implementation of antigen processing used in this work. How realistic is this displacement? T cell clones or hybridomas are able to respond in vitro to densities of the agonist peptides of less than a 1000 peptides per APC. This would correspond to displacements of less than 1% in the APC surface. If these same values were assumed sufficient to trigger an immune response in vivo the extent of displacement observed in our simulations would be very unrealistic. However, there are two arguments against this simple reasoning. First the studies referred above prove that T cells activation in-vitro is quite sensitive to the number of antigens at the APC surface. But even if the same sensitivity is expected for T cell activation in-vivo, the existence of cell activation does not imply the appearance of an immune response in-vivo. In our simulations there are many situation where effectors T cell activation and proliferation is observed but still there is not an effective immune response being mounted, since regulator keep effectors expansion under control. There may be need a higher level of expression of the foreign antigen at the APCs surface to overcome immune regulation, as predicted by our model. But to our knowledge so far there are no experimental estimates of this quantity. Second, and perhaps more significant each antigen is made of many different peptide determinants. Although individual peptides may represent a too small fraction it is not unlikely that the ensemble of peptides from a bacteria may displace a significant fraction of self-antigens. Moreover, in the case of an infectious antigen or antigens presented in adjuvants that promote inflammation, the physical displacement of the self-antigens by foreign antigen may synergize with alterations in pathways of antigen processing and presentation (Khan et al., 2001) that may result in cryptic peptides becoming dominant and dominant self-antigens becoming cryptic.

Following these considerations it is not unlikely that a foreign antigens leads to a significant reduction in self-marker presentation. Nevertheless, it is noteworthy that the relevant event is not so much the extent of this reduction but the sensitivity of APC-T cell conjugation and T cell activation processes to the change in agonist peptide fraction. The loss of self-antigens necessary to lose Regulators can be made very small as long as it is enough to entail a marked change in conjugation constants and T cell activation. It is reasonable to assume that conjugation processes are highly sensitive to changes in agonist peptide presentation. Many different molecules are involved in T cell-APC conjugation, participating in assembling and stabilizing the immunological synapse (reviewed in (Bromley et al., 2001)). The association of different receptor-ligand pairs in the synapse is likely to be cooperative. Also, T cell activation in vitro is hypersensitive to low numbers of agonist peptides per APC (Demotz et al., 1990; Harding & Unanue, 1990; Valitutti et al., 1995), and to changes in peptide concentration (Sousa & Carneiro, 2000). Therefore in our model, hypersensitivity in T cell activation gains a new interpretation based on its functional role in facilitating the immune responses.

Immune responses to pathogens can trigger autoimmune diseases (Benoist & Mathis, 2001). In our model the Regulator populations that prevent autoimmunity are lost during an immune response, and the reestablishment of peripheral tolerance after the response ceases requires the influx of new Regulators from the thymus. This suggests that infections might trigger autoimmunity more easily in athymic than in euthymic individuals. Also, the risk of autoimmunity might increase with age as the thymus involutes. Nevertheless, the incapacity of Regulator populations to persist during immune responses in the model results from treating the

periphery as a homogeneous well-stirred compartment. Future extensions to include multiple peripheral compartments may allow the persistence of Regulators during an immune response in compartments containing APCs where self-antigens have not been displaced by foreign antigens. These compartments may replenish with Regulators those compartments where an immune response took place, thus overcoming the strict need for thymus.

The simulation results present here enlightened the roles of thymic selection in the reliability and robustness of tolerance based linked suppression, and of antigen presentation in promoting immunity. These simulations are obviously limited in their realism, due to the effort for maximum simplification of the underlying processes. Some straightforward extensions may overcome some of unrealistic aspects such as instantaneous changes in the antigen concentrations, the step functions in thymic selection, and the isotropy of the peripheral compartment. Notwithstanding, the conclusions about a significant influence of the thymus and antigen presentation on the interplay between tolerance and immunity should be valid for any model based on linked suppression. These mechanisms may be coordinated with additional mechanisms of lymphocytes crossregulation refining the behaviour of immune system.

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6. Acknowledgements

This work was supported by Program Praxis XXI of the Ministério para Ciência e Tecnologia, Portugal (grant Praxis/P/BIA/10094/1998). JC was supported by Fundação para a Ciência e Tecnologia - Program Praxis XXI (fellowships BPD/11789/97). KL was partially financed by the Fundação Calouste Gulbenkian.

Appendix-A. Competition exclusion in the model.

The model studied in this work shows a competitive interaction between T cell clones. If simulations are started with a given set of Effectors and Regulator clones and there is no source of new clones, then any attainable equilibrium will have a single Regulator clone and a single Effector clone. These two clones are those having higher APC conjugation constant. We demonstrate that this is the expected solution by *reductio ad asurdum*. Assume there are two Effector clones in the final equilibrium (E1 and E2) having different conjugation constants K1 and K2 for the APCs. The equations for the equilibrium of these clones are obtained by using 4 as:

$$\frac{dE_1}{dt} = 0 = P_e \cdot Hyp\left[0,\ Rc' \cdot V, s \cdot V \cdot A, s\right] \frac{sA}{sA - Rc'} Ec_1 - \eta\mu \frac{s-1}{s}\frac{Rc'}{A} \cdot Ec_1 - d\,Ef_1 \qquad \text{A1}$$

$$\frac{dE_2}{dt} = 0 = P_e \cdot Hyp\left[0,\ Rc' \cdot V, s \cdot V \cdot A, s\right] \frac{sA}{sA - Rc'} Ec_2 - \eta\mu \frac{s-1}{s}\frac{Rc'}{A} \cdot Ec_2 - d\,Ef_2$$

Using then equations (5) in A1 and transforming you get:

$$d = \left(P_e \cdot Hyp\left[0, Rc' \cdot V, s \cdot A \cdot V, s\right] \frac{sA}{sA - rc'} + d - \eta\mu \frac{s-1}{s}\frac{Rc'}{A}\right) \cdot \frac{FK_1}{1+FK_1} \qquad \text{A2}$$

$$d = \left(P_e \cdot Hyp\left[0, Rc' \cdot V, s \cdot A \cdot V, s\right] \frac{sA}{sA - rc'} + d - \eta\mu \frac{s-1}{s}\frac{Rc'}{A}\right) \cdot \frac{FK_2}{1+FK_2}$$

Subtracting these equations we obtain:

$$0 = \left(P_e \cdot Hyp\left[0, Rc' \cdot V, s \cdot A \cdot V, s\right] \frac{sA}{sA - rc'} + d - \eta\mu \frac{s-1}{s}\frac{Rc'}{A}\right) \cdot \left(\frac{FK_1}{1+FK_1} - \frac{FK_2}{1+FK_2}\right) \qquad \text{A3}$$

Equations in A3 and A2 are true if and only if $K_1 = K_2$, which contradicts the postulate that the two clones had different conjugation constants. This result proves then that in this model at equilibrium only one Effector clones will persist or multiple undistinguishable clones with the equal conjugation constants. This property of the model is typically referred as competitive exclusion. We know demonstrate that the clone with highest conjugation constant is the only survivor. It is enough to assume that E1 survive and it is at equilibrium alone in the niche, determining a given value of the number of free APC sites F=F1. Then we introduce a very small number of E2 cells in the system, substituting the value f1 in the equation for the derivative of growing for the new clone, it can be easily checked that if K2<K1 then this derivative will be

negative perpetuating the assumed equilibrium but if K2>K1 then the derivative is positive and the clone E2 growth until it displace the clone 1 from the system.

The analysis for the Regulator clone needs to be different for different values of the switch parameter n in the model. In the case n=1 the model represent the situations where the regulatory cells are generated by converting the existent effector cells to the regulatory phenotype. In this variant of the model there is no competition among the regulatory clones per se. But as can be immediately realized from equations 4 in any potential equilibrium there will be only R clones that have a counterpart in effector clones and consequently have an influx of new cell for it. Then as there is competition among the effector clones, in any equilibrium containing R cells, there will be a single clone and this one is just the counterpart of the clone equilibrated among the E cells. In the case n=0 the model represent a situation where the R cells proliferate stimulated by interaction with the E cells. In this variant of the model there is actually competition among the R cells. The latter can be proved similarly to what we did for the E cells. Let us presume that two different R clones are simultaneously at equilibrium. Then the equations for this equilibrium would be:

$$0 = \mu \frac{s-1}{s}\frac{Ec'}{A} Rc_1 - d\, Rf_1 \qquad \text{A4}$$

$$0 = \mu \frac{s-1}{s}\frac{Ec'}{A} Rc_2 - d\, Rf_2$$

Substituting in A4 the relation in 5 and the subtracting the two equations we get:

$$0 = \left(\mu \frac{s-1}{s}\frac{Ec'}{A} + d\right)\cdot\left(\frac{F\,K_1}{1+F\,K_1} - \frac{F\,K_2}{1+F\,K_2}\right) \qquad \text{A5}$$

The relation A5 similarly to what happens to the equation A3 is only satisfied when K1=K2. So in any equilibrium where the R cells are not zero, there will be a single R clone in the system. This clone is the one with the highest conjugation constants to the APCs. Note that in this second variant of the model the competitive interactions among the R clones are independent of the competitive interactions among the E cells, so there is no obvious relation between the specificities that dominate among the E cells and among the R cells. This clearly contrasts to the previous model variant where at equilibrium these two specificities are always the same one, and the E cell clone with highest conjugation constant determines it.

Inverse Correlation for the Incidence of Autoimmunity and Infections predicted by a Mathematical model of T cell mediated suppression.

Kalet León[1, 2, 3], Jose Faro[2], Agustin Lage[1] & Jorge Carneiro[2]

[1]Centro de Inmunología Molecular, Habana, Cuba. [2]Instituto Gulbenkian de Ciência, Oeiras, Portugal.

[3]Author to whom correspondence should be addressed.

Abstract

The contribution of pathogenic infections to the etiology of autoimmune diseases remains one of the outstanding problems in immunology. According to the classical immunological concept of antigen mimicry, a direct correlation between the incidence of autoimmunity and infections would be expected. This view is supported by a few examples of autoimmune disorders, which are documented as being caused by infection with particular pathogens. In contrast, there are several experimental animal models where infection appears to prevent the onset of autoimmunity. Moreover, some epidemiological studies suggest an inverse correlation between the incidence of autoimmunity and infections in human populations. In the present study we use a theoretical model, previously developed to assess the role of regulatory T cells in natural tolerance, to suggest a solution to this puzzle. The concepts here developed delineate the conditions predicting an inverse correlation between the incidence of autoimmunity and exposition to common infections, and those in which antigen mimicry and inflammation of target organs have a role in the etiology of specific autoimmune disorders.

The increasing incidence of autoimmune and allergic diseases in wealthy countries with occidental style of life has been a clear and worrying trend in the last 50 years [1-3]. Possible causes for that trend range from changes in food and sedentary habits, to environmental chemical contamination to stress due to faster rhythm of daily life. However, it is difficult to quantify properly those factors, and hence how much they contribute to that trend cannot be easily estimated for correlation purposes in populations with different incidences of autoimmunity.

More recently, a different view has emerged that relates the incidence of autoimmune diseases to the relative cleaningness of the immediate environment[4,5]. Although there is no solid rational for this view, it is supported by two independent lines of evidence. On the one hand, there are several epidemiological reports of a north south/gradient for the incidence of autoimmune diseases in normal resident [6-9] and immigrating[10,11] human populations, which is inversely correlated with the prevalence of some infectious diseases [5]. On the other hand, there are several experimental animal models where the onset of autoimmune syndromes is prevented by particular infections[12,13,14] or by rearing autoimmune-prone animal strains in specific pathogen free instead of normal conditions[15,16]. As a whole, these observations hinted at a "hygienist" hypothesis[17] according to which common infections may have a role in preventing autoimmunity.

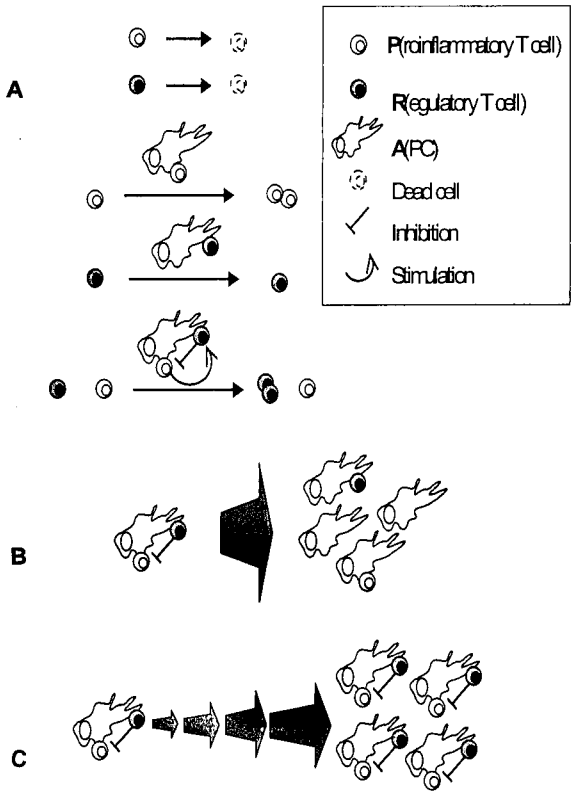

Fig 1. A model of T cell mediated tolerance and the effect of infections on autoimmunity. A, The basic interactions between regulatory and proinflammatory T cells driven by APC. B, Acute infections that cause a sufficiently large increase in APCs dilute "practically instantaneously" inhibition of regulatory cells on proinflammatory cells. C, The same increase in the numbers of APCs resulting from small incremental steps following encounters with common infections will allow the adaptation of regulatory cells population size and re-enforce tolerance.

Interestingly, the hygienist hypothesis seems to contradict the canonical immunological view of autoimmunity as caused by the cross-reactivity of a microbial antigen and some host-antigen in the course of infection (antigenic mimicry) [18] or as a consequence of pathogen-induced inflammation at the target organs [19,20]. These latter hypotheses would naturally predict a direct correlation between the incidence of autoimmunity and infections. Epidemiological studies have suggested such an association for some autoimmune diseases [7] and, further, antigenic mimicry has been documented in a few of these cases —notably, streptococcal miocarditis [21], Lyme arthritis [22], rheumatoid arthritis [23]. More deeper analysis revealed, however, that it is far from well established in most autoimmune disorders [24,25]. Nevertheless, the canonical view is also supported by different animal models in which specific, pathologic autoimmune responses are triggered by experimental infections or proinflammatory adjuvants (reviewed [26]); or in which [27] or iatrogenic [28,29] autoimmune syndromes are favored by comensal or infectious microorganisms.

The apparent paradox that infection may be causing and preventing autoimmune diseases demands a deeper understanding of the mechanisms mediating natural tolerance to host-antigens. Natural tolerance has been proposed to involve a continuous and active process, because clonal deletion in primary lymphoid organs is relatively inefficient in purging the lymphocyte repertoire of self-reactivities [30]. A paradigm of the basis for this active and continuous tolerance activity that is gaining rapidly a prominent status is that of regulatory T cells [31]. Special attention has been paid to CD4+CD25+ T cells [32]. These regulatory T cells are naturally generated in the thymus in a manner strictly dependent on MHC-Class II expression by cortical thymic epithelial [33], and they suppress the activity of other potentially pathogenic auto-reactive CD4+ T cells both in vivo and in vitro [34]. Recently, we have developed a mathematical model to assess the role of these regulatory cells in natural tolerance [35-37]. In the present study, the etiology of autoimmunity predicted by this model is explored. The results show that a view of natural tolerance based on regulatory T cells can provide a simple rational for an inverse correlation between the incidence of autoimmunity and infections, yet accounting also for the potential role of antigen mimicry in the etiology of some particular autoimmune disorders.

In our model three mutually interacting cell populations were considered[35]: (a) antigen presenting cells (APCs) displaying MHC-self-peptide complexes at the membrane; (b) proinflammatory autoreactive T cells (P) that can potentially provoke autoimmunity; and (c) regulatory autoreactive T cells (R), which suppress proinflammatory T cells. Interactions between P and R cells were postulated to require the formation of multicellular conjugates with APCs. Immunity and tolerance were interpreted in the model, respectively, as the competitive exclusion of R cells by P cell populations and the coexistence of both R and P cell populations at equilibrium. Several possible mechanisms for the interaction of the regulatory T cells and their targets at the APC surface were then compared on their capacity to account for the results of *in vivo* [35] and *in vitro* [36] experiments from the literature. Overall it was concluded that the suppressive mechanism requires, first, that R cells inhibit the proliferation of P cells, and second, that the persistence and growth of the R cell populations is

dependent on P cells (fig.1, panel A). (See Box 1 for a mathematical formulation of the model describing this mechanism). The study of this mechanism[35] demonstrated that three different dynamical behaviors are possible. Further, that study showed that the dynamical behavior set into play is determined by two key composite parameters, $G_R = K_R \mu A /d$ and $G_P = K_E \pi A /d$, which represent the "growth indexes" of R and P cell populations. These two parameters define, thus, three regions in the parameter space according to the predicted dynamic behavior (fig. 2). For parameter values in region I the system always evolves to an equilibrium state in which there are no R cells, irrespective of the initial proportion of P and R cells. Therefore, in this parameter region autoimmunity will always emerge in the system. Parameter region II is characterized by an equilibrium state in which the population of R cells controls the expansion P cell population, independently of the initial proportion of P and R cells. Here a stable tolerant state is always obtained. Finally, in parameter region III the system is bi-stable, meaning that either the state in which no R cells are present (autoimmune disease) or the state in which P and R cells coexist in stable balance (tolerance) can be reached depending on the initial proportions of P and R cells.

We have argued previously[35] that healthy immune systems necessarily operate on the bi-stable regime of region III. This is the only condition compatible with the observations that healthy animals have significant numbers of both R and P cells, and that the incidence of autoimmune pathology or tolerance can be modulated in recipients adoptively reconstituted with different proportions of P or R cells. Furthermore, when the model was further extended[37] to include many P and R cell sub-populations differing in their antigen specificities, it could be shown that only parameter values within region III lead to tolerance to host-antigens while retaining the capacity to mount specific immune responses to infectious agents. Moreover, in agreement to previous suggestions [38,39], this study indicates that a trade off between two opposite selecting forces determines the optimal relationships between parameters, which happen inside region III. On the one hand, there is the need to minimize the risk of autoimmunity, which in the model implies being close to the interface with region II. But, on the other hand, there is the need to minimize the susceptibility to infections, which in the model implies being close to the interface with region I. Thus, in this theoretical framework, the risk of autoimmunity is the price that must be paid for assuring immune responses to pathogens.

Autoimmunity can result from genetic defects, and indeed genetic associations have been largely observed in

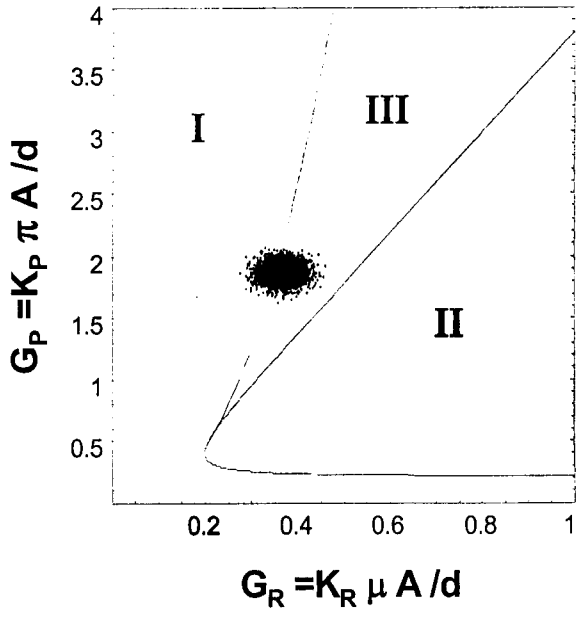

Fig 2. Bifurcation diagram with indication of a genetically diverse population of individuals. Areas labeled I, II and III correspond to those combination of values of G_P and G_R for which the model predicts, respectively, autoimmunity, total tolerance (including microorganisms) and tolerance only to host Ags. A Gaussian distribution for each growth index G_P and G_R in a population is assumed. The resulting 2D Gaussian distribution is here represented by plotting 10000 dots in the graph. The fraction of autoimmunity prone individuals corresponds to the fraction of the 2D distribution falling to the left of the line dividing the areas I and III

the etiology of autoimmune disorders. In the model, genetic factors should affect in a combined way the different parameters controlling essential properties of the system dynamics. Therefore, the different genetic variations in a given population will be reflected in effective variations of the parameters G_R and G_P on the individuals of the population. Let us assume that these two parameters are independent phenotypic characters distributed normally around respective averages $[G_R]$ and $[G_P]$, with variances $V[G_R]$ and $V[G_P]$. Then the fraction of individuals which are prone to autoimmune disorders can be estimated from the fraction of the 2D Gaussian distribution of the parameters G_R and G_p (dots in fig. 2) whose values are in the autoimmune region I. Given the fact that G_R and G_P are composite parameters, many different classes of genetic susceptibilities to

autoimmunity are identified by the model. Noteworthy among them are: 1) defects in T lymphocyte proliferation kinetics promoting either a higher division rate for P cells (large π) or a lower growth rate of R cell population (low values of μ); 2) defects in the death rates (d) of P and R lymphocytes; and 3) defects in the thymic selection affecting the antigen-specific conjugation of T cells with APCs that present host-antigens (larger K_P or lower K_R). The contribution of genetic factors to the etiology of autoimmunity in this model is most likely epigenetic. According to the model, a combination of several small defects in those factors can be equivalent to a single large defect in determining the propensity to develop spontaneously an autoimmune disease. However, the model also shows that variations in one genetic factor (*i.e.*, changes in the value of a given parameter) can compensate a variation in some other factor, leading to a normal phenotype.

Autoimmunity could also result from an extrinsic, contingent event breaking the otherwise tolerant status of an individual. In the simple model described in box 1, any event leading to a sudden (instantaneous) increase of the ratio between the number of APCs (A) and the number of T cells (P+R) will let the system to switch to the autoimmune state [35]. We have suggested previously two events of this kind that are biologically meaningful. The first case is that of an instantaneous reduction of P and R values keeping constant their proportion, which can be interpreted biologically as the induction of a lymphopenic status in a given individual. The second case consists in an instantaneous and sufficiently large increment in the number of APCs *A*, keeping *P* and *R* unchanged, which can be interpreted as the explosive increase of APCs presenting host-crossreactive antigens of microbial origin in the place of infection by recruitment, differentiation and proliferation. This property of the model provided thus a simple explanation for the reported emergence of autoimmunity in lymphopenic animals (reviewed in [40]) as well as for the induction of organ specific autoimmunity by local infections [41] and by immunisation with tissue-antigens or their microbial mimics in adjuvant (reviewed in [42]).

Infections that provoke autoimmunity may be quite rare. Common bacteria and viruses usually stimulate immune activity without provoking autoimmunity, perhaps due to the absence of antigenic mimicry or because they do not induce a sufficiently large inflammation. In any event, the frequent exposition of an organism to infections may naturally have different effects in the organization of its immune system. To model this effect, the particular way in which infections may affect the basal immune system activity to self-antigens has to be specified. Two biologically reasonable scenarios are analyzed in what follows, showing the conditions under which an inverse correlation between the incidence of infections and the incidence of autoimmunity is predicted by the model.

The first scenario considers that different microorganisms inside an individual host stimulate moderately the innate immune system, each contributing to the enhancement of antigen presentation by affecting the number of APCs and their metabolism. Most of the enhancement of presentation will be specific for microbial antigens but this will also augment, necessarily, the presentation of host-antigens. As an individual host develops and accumulates encounters with common microorganisms, the number of APCs presenting host-antigens will slowly raise by many small increments (see fig. 1C). Following this line of reasoning, living in an environment with a higher incidence of infections, will lead to a higher mean level of presentation of host-antigens in each individual. To model this hypothesis in the simplest way, it is assumed that the average number of APCs presenting host-antigens per individual (A) has a genetically determined basal level (A0) and increases proportionally to the incidence of infections, I, in the population (see item A in Box 2). Since composite parameters G_R and G_P are proportional to the number of APCs (A) in the system (see axes of fig 2), it follows that the point representing a given individual in the parametric space of figure 2 will change with the value of A along a straight line crossing zero. As a consequence, the assumed 2D-Gaussian distribution of parameters G_R and G_P in a host population will be displaced deeper into the parameter region III as the incidence of infection, *I*, increases in that population (see fig 3a) and consequently the frequency of autoimmune individuals will decrease. In other words, assuming that multiple and sequential infections induce an stepwise increase of the average number of APCs presenting host-antigens, the model predicts an inverse correlation between the incidence of autoimmunity and the incidence of infections (see fig 3b).

The second scenario assumes that during the course of infections there is a bystander stimulation of autoreactive T cells. Although the immune reaction to a given pathogen is mostly specific there will be always some activation of autoreactive T cells, both R and P cells, perhaps due to release of cytokines or to co-presentation of host and pathogen antigens by the same APC in a highly inflammatory context. So in practice common infections may induce an additional, bystander expansion of autoreactive P and R cell populations, which is not the

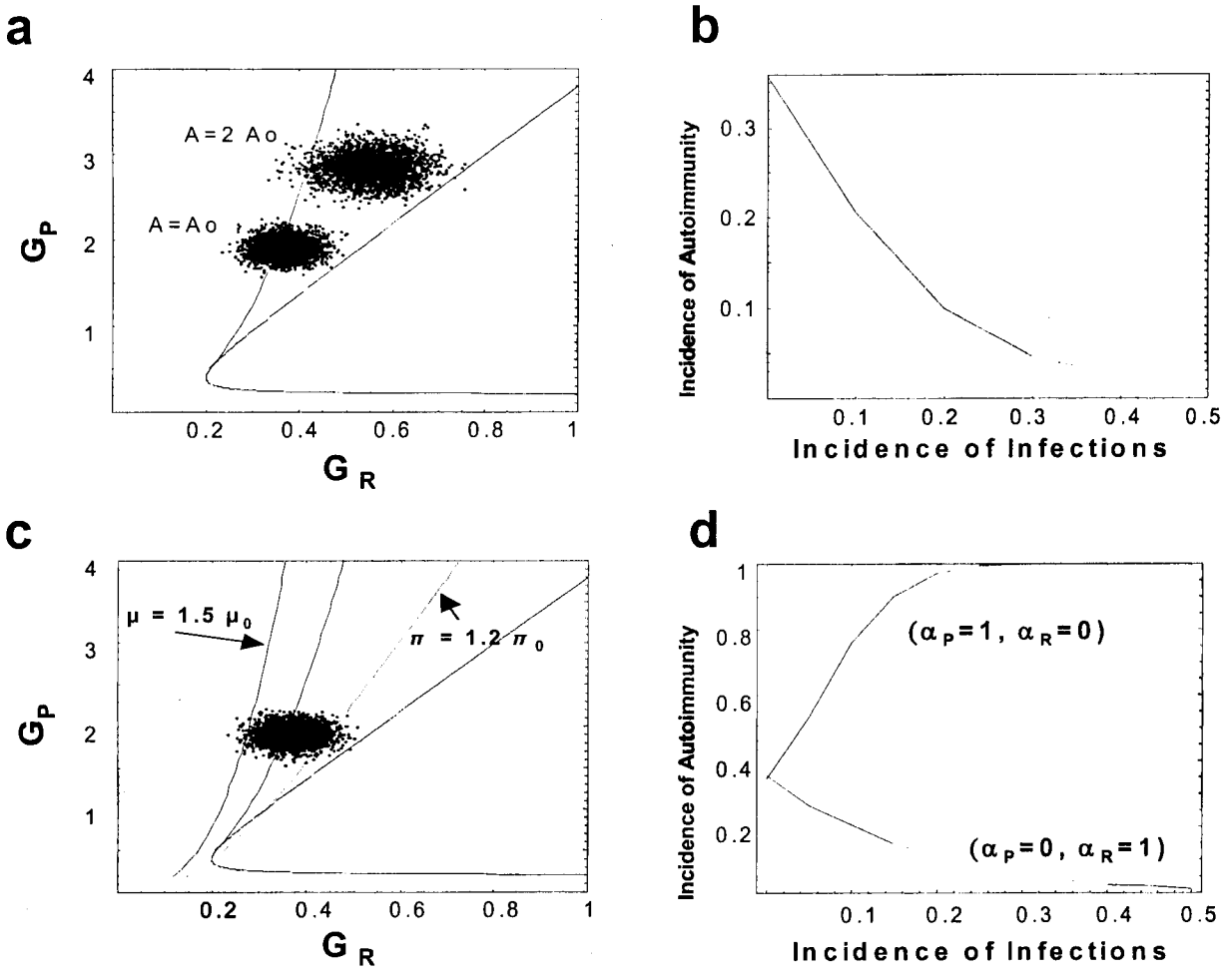

Fig 3.- Effect of common infections on the incidence of autoimmunity. a, A scenario in which common infections increase to double the average host-antigen presentation; b, expected correlation between the incidence of infections and the incidence of autoimmunity corresponding to the scenario shown in Panel a; c, a scenario in which the response to common infections increase the growth rate of either regulatory cells ($\alpha_R \times I = 0.5$ and $\alpha_P = 0$) or proinflammatory cells ($\alpha_P \times I = 0.2$ and $\alpha_R = 0$); d, expected correlation between the incidence of infections and the incidence of autoimmunity corresponding to the two cases shown in Panel c

result of the basal dynamics of their interaction with host-antigens (i.e in the absence of infections). This effect can be incorporated in the model by assuming that due to bystander stimulation there is an increase of the basal value of the growth parameters of the P and R cells (π_0 and μ_0 respectively) that is proportional to the incidence of infections I (see item B in Box 2). The growth indexes G_P and G_R will change thus linearly with the changes in π and μ, shifting some of the interfaces between parameter regions in the diagram of figure 2. To illustrate this, in figure 3c it is shown that if an infection stimulates the expansion of P cells but not R cells ($\alpha_e = 1$ and $\alpha_r = 0$) the interface between the parameter region I and those parameter regions where tolerance is possible (II and III) shifts to the right, expanding the autoimmune region in the model (fig 3c, dashed line). On the contrary, if infections stimulate the expansion of R cells but not that of P cells ($\alpha_r = 1$ and $\alpha_e = 0$) this interface shifts to the left, contracting the autoimmune region in the model (fig 3c, dotted line). Consequently, the model predicts that if frequent infections stimulate the expansion of P cells,

then the incidence of autoimmunity will correlate directly with the incidence of infection (fig. 3d, dotted line). Contrariwise, when frequent infections lead to the expansion of R cells the model predicts an inverse correlation between the incidence of autoimmunity and infections (fig. 3d, solid line).

We have analyzed here a model of T cell mediated tolerance, previously developed for individual hosts, in the more general context of the genetic/epidemiological domain at a population level. This provided a new theoretical way to approach the paradoxical observations that infections may elicit specific autoimmune diseases under some circunstances but that the average microbial load may protect (non-specifically) against autoimmune pathologies. Based in our present analysis, we suggest that the solution to this puzzle resides in the way microorganisms engage the individual hosts and alter the parameters determining the dynamics of regulatory and proinflammatory autoreactive cells.

These opposing consequences of host-microbial relationships stem in the model from the non-linearity of the mechanism underlying tolerance. Particularly relevant are the postulates that interactions between regulatory and proinflammatory cells require direct interactions while conjugated on the same APC (or more generically, while activated in the same inflammation-dependent niche), and that regulatory T cell populations depend on proinflammatory cells to grow. We notice that the second postulate is necessary but not sufficient because it will predict the protective effect of infections on autoimmunity but fails to explain how acute infections and inflammation could trigger autoimmunity. The later property requires that a fast increase in the number of APCs [35,36] or in the numbers and/or size of inflammation-dependent niches [43] will decrease the inhibitory interactions between regulatory cells and proinflammatory cells.

The genome of host populations may be adapted to common microorganisms present in the population's usual environment, by selection of alleles that compensate phenotypically the effects of microbial load and optimizes the trade-off between defensive immunity and tolerance. Populations adapted to different environments would be expected to have genomes differing in their contribution to parameters GP and GR. Thus, the basal, genetically determined values of GP and GR may be lower in populations adapted to environments with high infection prevalence and diversity than in populations living in environments with low infection prevalence or diversity. Recent immigrants from the first population would be more prone to autoimmunity in the "cleaner" environment of the second population. It is tempting to relate this scenario to the documented observation that the incidence of autoimmune pathologies is higher in immigrants African or Afro-Caribean origin in the United Kingdom than in the resident population [44]. Notwithstanding its potential interest, this hypothesis raises more questions than answers. For instance, what is the rate of adaptation of populations by negative selection on autoimmune individuals? Significant reduction of infection prevalence in Western Europe has taken place in the last century, raising the question on whether the rate of adaptation of resident populations is enough to justify the differences observed between the prevalence of autoimmunity in residents *vs.* immigrants.

Finally, genetic studies of autoimmune diseases typically implicate tens of candidate loci in a complex causal pathway [45-50]. Assigning a causal role to disease-associated genes is necessary to understand their effects and to design therapeutic approaches. This assignment requires the previous identification of a corresponding phenotypic trait. However, the association techniques are essentially silent in this respect. Our mechanistic model could have operational value in this task, since it identifies not only a few traits as being a priori the most likely candidates, but also the operational/mechanistic conditions that associate them to disease propensity or protection. For example, genes involved in lymphocyte proliferation or survival are obvious candidates. Furthermore, those alleles that increase lymphocyte growth indexes are good candidates for disease protection, while those decreasing growth are candidates for disease-propensity.

Box-1. A model of the dynamics and interactions of T cells involved in natural tolerance

The dynamics of proinflammatory P cells and regulatory R cells, which interact upon formation of multicellular conjugates with the APC, is modeled by the following equations:

$$\frac{dP}{dt} = \pi \cdot \mathrm{H}(0, R_c, s \cdot A, s) \cdot \frac{s}{s - R_c / A} P_c - d \cdot P_f$$
$$\frac{dR}{dt} = \mu \frac{s-1}{s} \cdot \frac{E_c \cdot R_c}{A} - d R_f \qquad (2)$$

with

$$\mathrm{H}(0, R_c, s \cdot A, s) = \binom{R_c}{0}\binom{s \cdot A - R_c}{s} \Big/ \binom{s \cdot A}{s} = \binom{s \cdot A - R_c}{s} \Big/ \binom{s \cdot A}{s}$$

where:

P, R Total number of P cells and R cells at any given instant t.

A Total fixed number of APCs.

P_c, R_c Number of P and R cells conjugated with APCs.

P_f, R_f Number of free P and R cells, given by $R_f = R - R_c$ and $P_f = P - P_c$

s Maximum number of T cells that can conjugate simultaneously with an APC.

d Death rate of P and R cells.

π Proliferation rate of P cells.

μ P cell-dependent proliferation rate of R cells.

P_c and R_c are obtained as:

$$P_c = \frac{F K_P}{1 + F K_P} P, \quad R_c = \frac{F K_R}{1 + F K_R} R \qquad (4)$$

where K_e and K_r are the conjugation constants of P and R cells with the APC respectively; and F is the total number of free APC sites, that is obtained by numerically solving the following equation:

$$F = s \cdot A - \frac{F K_P}{1 + F K_P} P - \frac{F K_r}{1 + F K_r} P \qquad (5)$$

Note that in the formulation of eqn.1 we assuming that a single R cell in a multicellular conjugated is sufficient to inhibit the proliferation of all the P cells in the same multicellular conjugate.

Box 2– Modelling the effect of common infections on T cell mediated tolerance.

There are two non-mutually exclusive scenarios by which common infections could affect the parameters in the model of T cell mediated tolerance described in Box 1:

A) According to the first scenario the average number of APCs, A, in each individual would be proportional to I, the incidence of infections in the population:

$A = A_o + \alpha \cdot I$

where A_o is the basal, genetically-determined number of APCs and α is a proportionality constant.

B) According to the second scenario, during responses to common infections there is a bystander stimulation of the growth of autoreactive regulatory and proinflammatory cell populations. To keep it simple, we assume that the average growth rates of P and R cells in each individual is incremented proportionally to I:

$\pi = \pi_0(1 + \alpha_P \cdot I)$ and $\mu = \mu_0(1 + \alpha_R \cdot I)$

where π_0 and μ_0 are either the basal, genetically-determined proliferation rates of P and R or the proliferation rate of P cells and the differentiation rate of R cells (both ways give equivalent results), and α_P and α_R are proportionality constants.

Acknowledgements

The authors would like to dedicate this work to the memory of the late Professor Mario Arala-Chaves. They also would like to thank their colleagues at the IGC, namely, Carlos Ferreira, John Stewart, Dan Holmberg, Werner Haas, and António Coutinho for many discussions that are at the origin of this work. This work was supported by Program Praxis XXI of the Ministério da Ciência e da Tecnologia, Portugal (grant Praxis/P/BIA/10094/1998). JC and JF were supported by Fundação para a Ciência e Tecnologia - Program Praxis XXI (fellowships BPD/11789/97 and BCC/18972/98, respectively). KL was partially financed by the Fundação Calouste Gulbenkian.

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Chapter 3

GENERAL DISCUSSION

3.1 Overview of this section.

Particular discussions have been conducted in each section of the chapter of results. Therefore here the detailed discussion of the individual problems studied in each chapter would be redundant. Instead, this final section will be devoted to highlight the main conclusions of the thesis as a whole unit. This discussion will be conducted in two main directions, given the bio-mathematical approach followed here. On the one hand we will look for immunologically relevant conclusions. So, we will ask how this Thesis enlarges the understanding of the dominant tolerance phenomena? And on the other hand we will extract conclusions from the mathematical modeling point of view. In other words, we will ask what was learned from the application of mathematical modeling techniques in this thesis? The following sections separate these two lines of discussion.

3.2 How this thesis enlarges the understanding of dominant tolerance phenomena?

As stated in the introduction, T cell mediated suppression is one of so called dominant mechanisms that contribute to tolerance to normal body components (i.e. natural tolerance) and it is in the very core of current studies in immunology. This Thesis provides the first comprehensive study, through mathematical modeling, of different aspects of these phenomena. Two complementary blocks of results are distinguished in the Thesis. The first block comprises the sections 2.1 to 2.3 and it was devoted to study the mechanism of interactions by which the regulatory T cells suppress their target cells. The second block comprises the sections 2.4 and 2.5, which built on top of the results of the previous block and studied the quantitative consequences of this suppressive interaction for the overall behavior of the immune system. Particularly we studied the conditions where this suppressive interaction is compatible with an effective self/non-self discrimination in the immune system and the implications of this interaction for the etiology of autoimmune disorders. The following sections discuss separately the main conclusions of this Thesis on the different aspects of T-cell mediated suppression.

3.2.1 About the mechanism of actions of CD4+CD25+ Regulatory T cells.

There are three main scaffold mechanisms for the interaction of regulatory T cells and their targets E cells on the current debate of immunologist (see figure 1). The mechanism A proposes that the R cells suppress the E cells by a direct R to E cell interaction once the R cells have been activated on their specific interaction with the APCs; Mechanism B proposes that the R cells produce some

soluble cytokine that suppress E cells activity; Mechanism D proposes that the R cells directly modulate the activity of the APCs, upon specific interaction, suppressing at least temporary their capacity to stimulate the E cells; And Mechanism C, which have been extensively study in this thesis, proposes that the R cells suppress the activity of the E cells while they are simultaneously conjugated with the same APC. Arguments in favor and against each one of these mechanisms have been drawn in the literature; so let us bring over this discussion highlighting how our results in section 2.1 to 2.3 contribute to it

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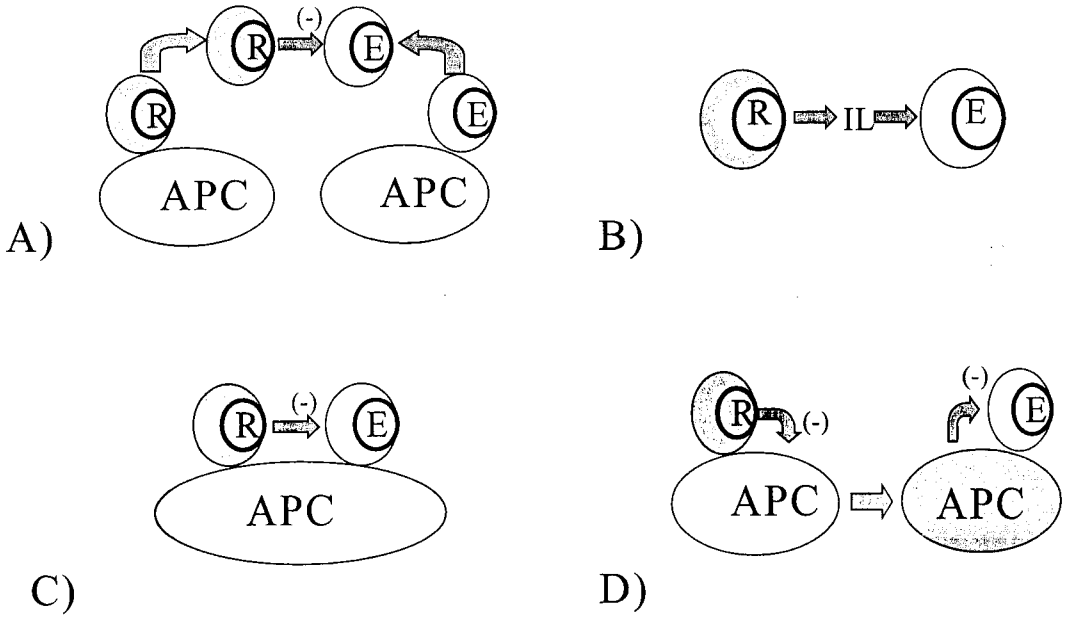

Figure 1. Scaffold mechanisms for the interaction between targets (E) and regulator (R) cells in T cells mediated suppression phenomena

The mechanism A has been supported by the group of Shevach et al with the results of *in vitro* culture [1] using CD4+CD25+ and CD4+CD25- cells from two TCR transgenic animals with antigenic specificities for the HA-peptide 110-119 restricted to the MHC allele I-E^k and the PCC-peptide 88-104 restricted to the MHC allele I-E^d. This study showed that CD4+CD25+ regulatory T cells from either of these transgenic animals could suppress the proliferation of the CD4+CD25- naïve T cells from the other transgenic if co-culture with a mix population of APCs expressing respectively the MHC alleles H-2k and H-2d plus the two peptides of interest. This experiment was interpreted as evidence for the mechanism A, which was then further documented in a second experiment, where the HA transgenic regulatory CD4+CD25+ T cells stimulated with plate-bound anti-CD3 plus IL-2 and subsequently washed were shown to suppress the proliferation of PCC transgenic naïve CD4+CD25- T cells stimulated with APCs plus the PCC 88-104 peptide. Despite

the latter results, it is difficult to accept mechanism A for suppression. Criticism came from the fact that the interpretation of the previous experiments relay on the assumption of no cross-reactivity of either the E or the R cells in the transgenic animal with the different peptides expressed on the different MHCs. This criticism is very pertinent, since the animals used are TCR transgenic in Rag-competent background and typically on this animals a considerable fraction of the total T cells express a non-transgenic TCR derived form endogenous recombination. Moreover in some experimental system, like in the MBP model developed by Lafaille et al, the regulatory T cells have been found mainly confined in the population of T cells expressing the endogenous TCR [2, 3] and the group of Shevack have previously reported [4] that HA specific R cells were not generated in a TCR transgenic animal in the SCID background where only HA specific T cells exist in the periphery. The latter analysis clearly cast doubts on the interpretation of the results reported by Shevach's group. A second argument against mechanism A is that it seems to contradict the result of several groups [4-6], which showed that the R cells do not to exert suppression on naïve T cells in cocultures stimulated with plate bound anti-CD3 antibodies. Note that suppression will be the natural expectation for the latter experiment, given mechanism A and moreover given that regulatory T cells becomes activated when stimulated with plate-bound anti-CD3 + IL-2 as reported by Thornton et al.[1] Finally there is also a theoretical argument against this mechanism A for suppression, relaying on the fact that it is antigen unspecific. Note that there is no specificity in the suppressive interaction between the E and the R cells, once the later cells have been activated. This lack of specificity is clearly counter intuitive given the specific character of immunological tolerance and it can be only resolved within this framework by assuming locality on the interactions. In other words the activated R cells cannot go far away without reverting to an inactive phenotype in which they need further activation on the APC to exert suppression, so they will only suppress the E cells being activated in their close environment. The latter assumption will also render mechanism A compatible with the concept of linked suppression and it clearly requires experimental validation. But moreover under this condition the dynamical properties of mechanism A will tend to resemble those ones of mechanism C, in the limit when the activated R cells loose their suppressive capacity so fast that it only manage to effectively suppress the E cells that have got activated next to it in the same APC.

The mechanism B for suppression, interaction mediated by a soluble factor, remains controversial in the field. In section 1.1.4.5 of the introduction we reviewed a set of experimental observations that shows a role for cytokines in T cell mediated tolerance in some *in vivo* systems, but also cast doubts on whether cytokines are a direct soluble mediator of the suppression exerted by the

regulatory T cells. Perhaps the only possibility still open for mechanism B is that suppression is mediated by an extremely short range acting cytokine, that is degraded or consumed so rapidly that we cannot detect it's activity in the in vitro cultures. i.e. this cytokine acts in such short-range that its suppressive activity cannot be observed *in vitro* suppression assays using transwell cell culture. Under this condition, mechanism B will stand for a juxtacrine suppressive mode of interaction mediated by a soluble factor, rather than the usual paracrine mode of interaction and its overall dynamical properties will tend to resemble those of mechanism A.

The mechanisms D and C are quite related to each other. Mechanism C can be seen as the extreme of the mechanism D where the R cells only render the APC inactive while they are physically conjugated to them. In other words the mechanism C is the limit of the mechanism D when the time the APC stays suppressed after detaching from the R cell is practically zero. The dichotomy of mechanisms D and C have been actually motivated by an analogy with the mechanism of cell cooperation between the helper T cells and the cytotoxyc T cells. A mechanism of helper-cytotoxic T cells cooperation like mechanism C was proposed by Mitchitson et al, despite some theoretical concerns with the potential inefficiency of this type of interaction, which require a three cell interaction [7]. More recently, several reports [8-10] indicated that helper-cytotoxic T cell cooperation proceeds by a mechanism like the mechanism B, where the APC work as temporal bridge between the helper T cells and the cytotoxic T cells. In the context of the suppressive interaction mediated by CD4+CD25+ regulatory T cells, mechanism D has gained interest following the observation that these cells modulate the expression of some costimulatory molecules by APC surface *in vitro* [11-14]. This mechanism, however, lacks direct demonstration, and is difficult to reconcile with the fact that suppression is typically observe in *in vitro* cultures using fixed APCs [5], where an active modulation of the APC activity is difficult to envisage. On the other hand the mechanism C on T cells suppression appears as an obvious consequence of the concept of linked suppression and its only criticism remains the same concerns raised by Mitchitson et al [7, 15] about the efficiency of interaction requiring the formation of multicellular conjugates.

By the time we started working in this Thesis the mechanism C was quite popular among immunologist, perhaps due to the impossibility to properly demonstrate any of the other two, which still remains nowadays. Honestly we start working in a model of this mechanism for suppression aiming to disprove it based on its presumable quantitative incompatibility with the result of *in vitro* suppression assays. Our results however, first make explicit the limitations in suppression efficiency intrinsic to this mechanism, but second convinced us that despite this inefficiency the mechanism

was still quantitatively compatible with the experimental results. In the way to this conclusion several other interesting results were obtained, that enlighten our current view of the mechanism of T cells mediated suppression.

The first of this result is the development of a mathematical model for T cells mediated suppression phenomena. This model could be a useful tool in the hand of experimentalist to analyze quantitatively the results of *in vitro* suppression assays. In our view this model can be especially useful to analyze the compatibility among experimental data coming from different groups. We provide a practical example of how this could be done in section 2.2. There we find, just by using the model, that the experimental data of *in vitro* suppression assays reported in the literature by different groups lay on a very similar curve if represented as the inhibition index as a function of the number of R cells per APC in the culture. This property was further confirmed by the experiments in section 2.4, where we actually used it as a good indicator that our culture were properly reproducing the results of other group and consequently we were observing the same phenomenon. Interestingly the same type of quantitative analysis raises an intriguing issue on the comparison of the regulatory activity of CD25+CD4+ T cells from mice [4, 5] and human [16-18] origin. The in vitro suppression assay with human T cells requires 10 times more regulatory T cells per APC than their mouse derived counterpart to recover an equivalent level suppression. Such differences on the efficiency of suppression may reflect just a bad optimization of the cultures conditions with human cells or that regulatory cells in humans are just a subpopulation among the CD25+CD4+ T cells [19], but they may also say that suppression reported on those experimental system are mechanistically unrelated to the result in mouse models.

Other important results of this Thesis relate to the existence of more subtle divergences of immunological thinking about the mechanism of suppression beyond those ones expressed by the scaffold mechanism A, B, C and D outlines above. Note that, even if, as we did, you assume valid mechanism C, still you can postulate many different detailed mechanisms for the regulatory interactions. For instances regulatory T cells can be assumed, according to different authors, to just deliver a suppressive signal to the E cells at the APC surface [6, 20] or to induce the E cells differentiation to the regulatory phenotype [21, 22]. Alternatively suppression may result from the simple competition of E and R cells for activation resources produced by the APC, with no particular R to E cells intermediary signal. In this sense, four alternative candidate models for the detailed mechanism of suppression were made particular in the model and compared on their ability to explain the results of *in vivo* (section 2.1) and *in vitro* (section 2.2) data in the field. Concluding,

from these two independent lines of results, that a valid mechanism of suppression requires that the R cells deliver a suppressive signal to the E cells, while the E cells stimulate the growth of the R population, either by proliferation of the R cells or by conversion of the E cells to the regulatory phenotype. These results effectively narrow down the number of candidate mechanism for suppression, discrediting some of the mechanism discussed in the literature.

Moreover we further study the mechanism, originally proposed in this Thesis, where the R cells are stimulated to proliferate by its interaction with the E cells at the APC. In collaborative work with the group of Jocelyne Demengeot (section 2.3), experimental support was actually provided for this mechanism, reporting for the first time that the regulatory T cells proliferate *in vitro* when coculture with the E cells. Despite this result the proposed mechanism is far from being proved. More combined theoretical and experimental work is required in this sense. Note nevertheless that this mechanism where the E cells work as a growth factor for the R cells is quite reasonable, since it would provide a robust mechanism for regulation. Particularly we envisaged this stimulation of the R cells proliferation by the E cells as mediated by a soluble factor produced by the E cells, raising the hypothesis that IL-2 is such a soluble mediator. The latter hypothesis is based in the experimental facts that the R cells unable to produce Il-2 but they proliferate strongly if externally supplied with this cytokine [23], which is indeed produced by the E cells upon activation. Theoretically this hypothetical mechanism provides a physiological explanation for the expression of the CD25 marker in the Regulatory T cells, since this molecule is nothing but the high affinity chain of the IL-2 receptor. Moreover this mechanism provides an explanation for the disturbances to natural tolerance and homeostasis observed in animals mutant of the IL-2 or IL-2 receptor genes [24-27], based on their impact for the maintenance of regulatory T cells populations [28-30]. But testing experimentally the latter hypothesis is an issue for further development of the present work.

Summarizing, concerning the mechanism of suppression our results in sections 2.1 to 2.3 contribute: 1) to show the quantitative inefficiency of a suppressive mechanism requiring the formation of multicellular conjugates, but disregarding it as an impediment to successfully explain the existent experimental data; 2) to provide a mathematical model to quantitatively analyze the result of *in vitro* suppression assays; 3) to narrow down the number of candidate mechanism for suppression from those existent in the literature; and 4) to raise a testable hypothesis about the physiological role of IL-2 and IL-2 receptors in T cells mediated suppression phenomena.

3.2.2 Linked suppression and self/non-self discrimination.

Regulatory T cells suppress their targets cells, by a mechanism that is not mediated by soluble factors and requires linked recognition of specific antigens in the same APC (see section 1.1.4.5). The main implication of this mechanism of interaction is the lack of strict specificity in suppression. Note that a given regulatory T cell will suppress any T cell that recognizes the same APC (in mechanism D and C discussed in 3.2.1) or those cells that get activated on its proximal environment (in mechanism A discussed in 3.2.1), independently of whether or not they recognize the very same antigenic peptide at the APC surface. So the specificity in suppression is just partial and is linked to those peptides, which are simultaneously presented in an APC or in the same proximal environment. This sort of partial specificity in suppression is quite relevant in a theoretical framework for the overall behavior of the immune system. On the one hand, this property may guaranty that natural tolerance can be achieved with a relatively small number of Regulatory T cells. There is no need of having an expanded population of R cells for every existent self-antigen in the body. It is enough to have R cells, which are somehow tissue specific, since they can recognize a sort of "self-marker antigen" and ensure tolerance to all the remaining antigens in the same tissue. On the other hand, the lack of specificity in suppression raises a major theoretical problem to explain an effective self/non-self discrimination in the immune system (see section 1.1.5). Foreign antigens are always presented in APCs that also present some self-antigens; therefore the tolerance to these self-antigens may prevent the immune reaction to the foreign antigen too. In section 2.4, the latter problem was explicitly addressed, in simulations of the populations dynamics of multiple T cell clones containing both R and E cells, which interact to a set of antigens always present both in the thymus and in the periphery (self-antigens) and a set of antigens (foreign antigens) that become available in the periphery once the systems has equilibrated with the self-antigens. Overall our results show that under certain conditions the model could systematically tolerate the self-antigens while reacting specifically to the introduction of a foreign antigen. In other words, that an effective self/non-self discrimination could be indeed obtained, despite linked suppression and even when R clones are generated in the thymus with no particular bias towards a preferential recognition of self-antigens. This conclusion is certainly the major result of our theoretical study of the phenomena of T cells mediated suppression, but lets discuss closely here the etiology of the self/non-self discrimination observed in our simulations and the key assumptions, parameter conditions and limitations of the analysis.

Section 2.4 reports that the system reacts equally well to the change in antigen concentration for a self-antigen and for a foreign antigen, if the number of R cells that specifically recognize its derived-peptides is small enough and the raise in the level of presentation by APCs of this antigenic-peptides is large enough; tolerance to a given antigen is mainly characterized in the system by the existence of a relatively large number of specific R cells that recognize it. Moreover, preliminary results not reported in Section 2.4 add to these requirements for inducing an immune reaction in the system that the raise concentration of the given antigen need to be fast enough. The word "fast" means in this context a process that take place at most in the same time scale than a typical immune reaction, which is around a week; therefore if the antigen concentration is sufficiently raised in less than a week there could be an immune response, but if it takes a month then it will eventually induce tolerance. Overall in our simulations the system reacts to a sufficiently sudden and large enough change in the level of presentation by APCs of a given antigen that is specifically recognized by a low number of R cells, disregarding whether or not this antigen is a normal body component. Two features made self-antigens to be dynamically tolerated in the system. First their presence in the thymus, which ensures a positive selection of specific R cells with high-enough frequency; and second their persistence at the periphery, which provides a survival signal to their specific regulators. In other words, Tolerance to self-antigens is dynamically achieved primarily through the appropriate generation and selection of regulatory T cells in the thymus and it is then maintained by specific antigen recognition at the periphery

The basis for self/non-self discrimination described above is radically different to the one delineated in the theories described in the introduction, allowing this theory to overcome the main difficulties pinpointed there for each one of these theories. First of all in this view, natural tolerance is a dynamic process, kept by a continuous activation of lymphocytes, just as expected in dominant tolerance theories and in opposition to the expectation of recessive theories of tolerance. But moreover the immunogenicity of a given antigenic challenge, in this framework, is determined by three key dynamical properties of the particular challenge, which are the number of R cells recognizing the challenging antigen, the level of representation at the APC surface of the peptides derived from the given antigen and the kinetic of the change in the antigen concentration; Therefore they are not exclusively determined by the existence of T cells specific to the given antigen, as in the clonal selection theory (section 1.1.3.1), or by the preexistence of the antigen to the immune system organization as in the two signal model (section 1.1.3.2), or by the capacity of the challenging antigen to activate the innate immune system, as in the innates based theories (section 1.1.3.3). Actually the identification of the lately referred dynamical properties is an important result

of this work, since they provide a criterion for the interpretation of experimental data in this theoretical framework. In a broad sense, immunogenicity and tolerance in this theory are determined: 1) by repertoire selection, which in turn influence the number of R cells recognizing a particular antigen; and 2) by the way the particular antigenic challenge is performed, which in turn relates to the level of the antigenic peptides displayed at the APC surface and the kinetic for the change in the antigen concentration in the system. An illustrative way to represent the latter criterion is given in the figure 2, where a region for immunogenicity of a given antigenic challenge is defined as the upper zone in a three dimensional space with axes corresponding to: the number of R cells (Ro) that specifically recognize the antigen in the system; the level of presentation at the APC of peptides derived form the antigen of interest (po); and the time (To) the antigen takes to raise its concentration to guarantee the level of presentation given by po.

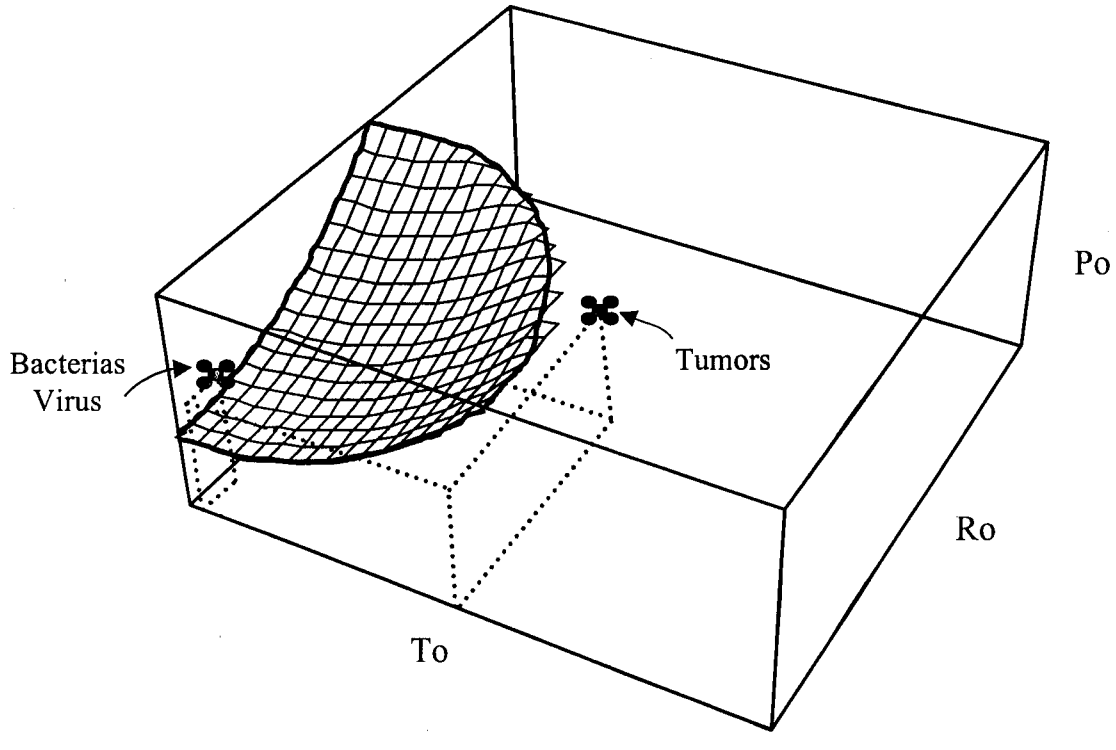

Figure 2. Schematic representation of the three key properties of an antigenic challenge, which determine its immunogeneicity, according to our model. The surface depicted separates this imaginary space on the immunogenic region above the surface and the non-immunogenic region below the surface. Some classical antigenic challenges are located in this space, just following common sense (see text).

Some classical type of antigenic challenge on the real immune system can be then placed in this space just using the common sense, as shown in figure 2, illustrating that their immunogenicity can

be easily explained in this theoretical framework. In the one hand, bacterial or viral antigens are most of the time sufficiently different from self-antigens on antigenic composition (more relevantly different from the tissue self-markers antigens) as to be recognized by a low number of R cells; and they replicate fast enough as to reach in a short time a considerable large value of po for its associated antigens. These properties situate them in immunogenic region of the figure 2. On the other hand a tumor is similar, but not identical on antigenic composition to the self-antigens, so it will be recognized by a relatively large number of R cells in the system. Moreover they grow relatively slowly, so they will take a longer time to reach a large level of presentation for its associated antigens. These properties will definitely situate most tumors in the non-immunogenic region of chapter 2.

A key element for all the results outlined above is what we referred in section 2.4 as the displacement hypothesis. This hypothesis presumes that the introduction of the foreign antigen in the system significantly reduces the presentation of self-antigen peptides by the APCs. This hypothesis naturally emerges from the fact that APCs display a limited number of MHC-peptides at their surface. Therefore the only way to display a new peptide is to displace some of the peptides previously exhibited at the APC surface. This assumption proposes a simple dynamical solution to the problems posses by linked suppression. A significant displacement of the self-antigen peptides from the APCs surface, reduce the capacity of self-reactive Regulatory T cells to bind and to get activated at the same APC than the foreign-antigen specific Target T cells, therefore it reduce the negative effects of linked suppression for the immune reactivity. At first sight the displacement hypothesis, could seem quantitatively unrealistic for immunologists. This reaction is mainly due to the results of several experimental system where the T cells clones have been shown to get activated or produce cytokines *in vitro* when stimulated with APCs that contain as few as 100 to 1000 copies of its specific peptide at the surface, The naïve extrapolation of this quantitative estimations as the number of specific MHC-peptides required to trigger an immune reaction *in vivo* would clearly rule out the displacement hypothesis. But as discussed in section 2.4, the experiments mentioned above provide information on the quantitative requirement for the activation of T cells *in vitro*. *In vivo* the antigenic threshold to achieve T cell activation may be much lower than the thresholds required to overcome active immune regulation and consequently mounts an immune response. The experiment required to prove or disprove the displacement hypothesis needs to be performed *in vivo* where linked suppression may be operative. Unfortunately, to our knowledge, no experiment of this kind have been performed so far, although we hope that the relevance of the issue uncovered by the present studies will encourage some experimentalist to assess it. Our expectation is that in order to

induce an effective immune response *in vivo* the foreign antigen peptides should occupy at least a 10 % of the total MHC displayed at the APC surface.

Another important aspect of the results in section 2.4 is the parameter conditions to obtain a biologically meaningful behavior in the simulations. Particularly interesting in this sense is the role intrathymic repertoire selection. Thymus has received special attention in the studies of dominant tolerance field, since it has been demonstrated as the primary source for the generation of CD4+CD25+ regulatory T cells. Lately many authors have concentrate on demonstrating that the generation of regulatory T cells is antigen driven (reviewed in [31]), being their results consistent with the hypothesis originally raised by Modigliani et al.[32, 33], where the generation of R cells was postulated as mediated by high affinity recognition of self-peptides in the thymus. This hypothesis is biologically reasonable and it is motivated by the intuitive consideration that having in the periphery R cells specific for the self-antigens and not for the foreign antigens would benefit an effective self non-self discrimination in the immune system. Note, nevertheless that the latter hypothesis will only reduce the specific component of suppression i.e. the effect of R cells that recognize specifically a given peptide; but it says nothing about the limitations that linked suppression may impose to this seminal problem i.e. the effect of R cells that act via unspecific linked recognition. To assess the latter problematic we have set up our *in silico* thymus to generate regulatory T cells unspecifically, looking to derive the implication of this assumption in the system. These simulations demonstrated that an effective self/non-self discrimination could be achieved even in these conditions, proving that Modigliani's hypothesis is neither a necessary nor sufficient for a proper self/non-self discrimination, although it indeed facilitates immune responses in the simulations (unpublished results). Despite this fact, our simulations evidence a crucial role of intrathymic selection of clonal specificities to achieve an effective self/non-self discrimination in this system. Particularly the simulations required that a sufficiently large number of R and E cells is exported to the periphery per time unit, bearing affinities for the self-antigen peptides inside a given windows. In other words, both positive and negative selections were identified as necessary conditions for a biologically meaningful behavior in this theoretical framework. Positive selection was required to guarantee the generation of a sufficiently high frequency of self-recognizing R cells, as to initially organize a tolerant state in response to the self-antigens; and negative selection was required to avoid the emergence at the periphery of very high affinity self-antigen specific R cells that will make the tolerant state so robust that could not be broken by the introduction of a foreign antigen. Therefore we proposes a novel role for the process of positive and negative selection in the immune system that is actually quite the opposite of the one related by the most

classical immunological theories. Classically positive selection should guarantee immune reactivity and not tolerance, by ensuring the right MHC restriction, while negative selection should guarantee tolerance and not immune reactivity, by deleting self-reactive clones in the thymus. Such a reversed view of the very same phenomena just substantiates that, as stated in the introduction, dominant tolerance and linked suppression represent a major shift in current immunological thinking. But what is actually most interesting in the latter result is the requirement for negative selection for regulatory T cells in the thymus, which has been so far included in most theoretically construct in the field of regulatory T cells with no obvious theoretical explanation. Our result provides such explanation as consequence of the linked character of suppression. If suppression is specific then there is no point in limiting the thymic generation of very high affinity self-reactive R cells, since they will just enhance tolerance to this particular self-antigen that you need to tolerate anyway. But if suppression is only partially specific and act by linked recognition, then this very high affinity self-reactive clone will prevent immune response to many different antigens including the foreign antigens, affecting the actual system reactivity.

Finally it is worth pinpointing here the limitations of the study reported in this thesis for the problem of self non-self discrimination. The major limitation of our analysis in section 2.4 is that every self-antigen in the simulation is equally expressed in the Thymus and the periphery i.e. we have neglect the existent of extrathymic antigens. We opted for simplifying the analysis, reducing the number of parameter in the system, following the rational of modeling as self-antigens only those tissue self-marker antigens that are present in the thymus and may be responsible for the tolerization of the remaining self-antigens in the same tissue. Nevertheless the issue of the extrathymic antigens deserves further theoretical studies in this system. We are currently performing new simulations to address this issue and preliminary results indicate that indeed tolerization of extrathymic antigens can be derived exclusively from the intrathymic presence of some self-marker antigens, but a still not fully established relationship need to be guaranteed between the cutoff affinities for positive an negative selection in the thymus and the relative levels of expressions in thymus and periphery of the self-marker antigens. Moreover the latter results can be complemented, following the classical conception of a neonatal window for tolerance. Following this line of reasoning there is a period in the ontogeny of the immune system where tolerant behavior is favored and where the tolerance to most extrathymic self-antigens is established. This favoring of tolerance can be envisaged in our model in many different ways. For instance assuming in this neonatal period a higher thymic output of both R and E cells; or an enriched production of R cells; or if following the proposal of Coutinho et al [33] some especially cross-reactive germ line

encoded R cell clones are assumed to be preferentially generated by the thymus. Note nevertheless that favoring tolerance does not mean that immunity is not possible. Actually the experimental results of Medaware et al [34] and Le Douarin et al [35, 36] demonstrate that most peripheral tissues, but lymphoid tissue and thymic epithelium are rejected if transplanted from an allogenic source, even in the neonatal period. Interestingly the two tissues that in these experiments are indeed tolerated and moreover induce tolerance to other peripheral tissues, have in common their presence in the thymus; therefore they can effectively interfere on lymphocytes selection, particularly by expressing those self-markers antigens relevant for the tolerance of peripheral tissues, just as expected in the above delineated theoretical framework. Alternative a solution for the problem of extrathymic antigens, perhaps relevant to those antigens that change its level of expression in periphery like hormones, appears when the change in antigens expression is sufficiently gradual. According to the discussions above, if the extrathymic antigens take months to increase significantly their expression in the body they will naturally induce tolerance in the system, with no requirement of further assumptions in the model. This latter idea is very interesting since it provide a simple explanation for the tolerizations of those extrathymic self-antigens, which may start to be expressed latter on in life. For instance some self-antigens associated to the hormonal changes during puberty for both male and females or to the hormonal changes during pregnancy for the females.

Summarizing the studies on this Thesis, particularly on section 2.4, explore the limitation that linked suppression imposes to an effective self non-self discrimination in the immune system. We first propose a quantitative solution to the latter problems based on the dynamic properties of the suppressive interactions. We then characterized the etiology of the self non-self discrimination observed in this theoretical framework, further deriving its implications. In the one hand we identify a tight relationship of the processes of positive and negative selection in the thymus and the capacity of the model to display a biologically meaningful behavior. Particularly positive selection was required to guarantee tolerance to self-antigens, while negative selection was requires to prevent that linked suppression turn off every immune reaction to foreign antigens in the system. In the other hand the three keys conditions that would render immunogenic a given antigenic challenge in this system were identified, providing an operational criterion to analyze experimental data in this theory. These conditions are 1) that the challenging antigen is recognized by a small number of R cells; 2) that this antigens reach a sufficiently high level of presentation at the APC surface; and 3) that the change in antigen concentrations associated with the given challenge is fast enough.

3.2.3 T cell mediated suppression and the etiology of autoimmune diseases.

Autoimmunity is the reverse face of tolerance and particularly of natural tolerance. In most classical immunological theories, which have a recessive view of tolerance, autoimmunity is explained as caused by a mistaken activation of lymphocytes. For instance autoimmunity is explained, in the clonal selection theory (section 1.1.3.1) by an error in thymic deletion that leads to the existence in periphery of an auto-reactive lymphocyte; in the two signal model (section 1.1.3.2) by an erroneous activation of auto-reactive T helper cells; and in the innate immune system based theories (section 1.1.3.3) by the erroneous activation of the innate immune system provoking inflammation in the response to a particular self-antigens. The most classically accepted cause for autoimmunity stands for the infection of an organism with a parasite that bare antigenic homology with some particular self-antigen in the body. This etiology for autoimmunity is typically referred as antigen mimicry and it has been properly documented in few cases [37] of known autoimmune disorders. The concept of dominant tolerance, more particularly T cells mediated suppression represents necessarily a change in the immunological view of autoimmunity. In this theoretical framework autoimmunity cannot be exclusively caused by erroneous lymphocyte activation, it is additionally necessary to overcome the existent tolerance for the self-antigens determinant. Particularly autoimmunity can be conceived in two major ways: 1) from a genetic defect that renders inefficient the mechanism of immune regulation; and 2) from a contingent event that produce a breakdown of the existent tolerant status.

In the studies in section 2.5 we have use our mathematical models of T cell mediated suppression developed in section 2.1 to investigate the etiology of autoimmune diseases in this theoretical framework. Our result identifies different potential causes for autoimmunity induction. First of all there are genetic defects that are predicted by the model to provoke tolerance disruption and consequently autoimmunity. These are: 1) Defects in T lymphocyte proliferation kinetic that promote either a higher division rate for the E cells or a lower growth rate for the R cells; 2) defects in lymphocyte death kinetic, that could affect differentially or not the E and R cells in the system; and 3) defects in the selection of lymphocytes specificities in the thymus, affecting the conjugation affinities to self-antigens in the population of E and R cells in the system. Interestingly, the contribution of these factors to the etiology of autoimmunity in the model is most likely epigenetic. On the one hand, variations in one genetic factor can compensate variations in some other factors, keeping normal a particular individual; and on the other hand many different combinations of small defects on these genetic factors could determine the propensity to autoimmunity. The model also

explains the induction of autoimmune diseases by antigenic mimicry, just as proposed by classical immunological theories, being additionally consistent with the result of experiments in several animal models were autoimmunity can be induced by immunization of the appropriate antigen plus adjuvant (reviewed in [38]). Moreover, the model explains the induction of autoimmunity generated upon treatments inducing lymphopenia and the quite particular role of thymus in this phenomenon (reviwed in [39]). This finding is particularly relevant since the latter etiology for autoimmunity is hard to explain in the context of the classical immunological theories, but it is naturally explained in the theoretical framework studied here. But the most interesting result in our studies of autoimmunity is the demonstration, that under very simple assumptions our model can predict an inverse correlation between the incidence of autoimmune diseases and the incidence of common infections. This result is quite striking and it allows the model to explain several experimental results, which are clear anomalies in the context of classical immunological theories. On the one hand it explains the results of epidemiological studies suggesting such inverse correlation between the incidence of autoimmunity and infections [40-44], particularly demonstrating that this phenomenon is not necessarily incompatible with the concept of antigenic mimicry. On the other hand this result may explain the result of several experimental animal model, where the onset of autoimmune disorders have been prevented by infection. Notable examples of the latter are the prevention of Lupus in (NZBNZW) F1 mice by infection with LDV [45], the prevention of diabetes in NOD mice by the infection with *mycobacterium bovis* [46] and *mycobacterium avium* [47], and the widespread observation that specific pathogen free NOD mice [48] and BB rats [49] show a much higher incidence of diabetes than conventionally reared animals.

Summarizing our studies in this Thesis provided a theoretical rational for the etiology of autoimmune disorders, which is more complete than the one provided in classical immunological theories. Particularly this study identifies several genetic defects and two major classes of contingent events, which in the model lead to autoimmunity, providing simple explanations for some interesting experimental data. Moreover the model provides a simple rational for an inverse correlation between the incidence of autoimmunity and infections, yet explaining the potential role of antigen mimicry in the etiology of autoimmune disorders.

3.3 About the mathematical modeling in this thesis

In this thesis mathematical modeling techniques have been applied to study different aspects of dominant tolerance and more particularly of the T cells mediated suppression. This fact exemplify

first of all, that as stated in section 2.2, mathematical models are built to ask precise questions, to address particular problems; therefore their main conclusion should be relevant for the analyzed problem. The latter should be the main and first criteria to evaluate the mathematical models in this Thesis, remembering in any case that the major value of a model, like the one of theories is not to be right or wrong, but to raise new testable hypotheses that promote and guide new experimentation in the field. We hope that the previous discussion in this Thesis has convinced the reader of the relevance and pertinence of our biological conclusions. Nevertheless there are some interesting aspects of the application of modeling methodology in this Thesis that deserves further discussion here.

3.3.1 A new mathematical model for the interactions in high order conjugates.

Section 2.1 developed, what to our knowledge is the first mathematical formalism to model the dynamic of several cell populations whose interactions depend on their simultaneous conjugation to a third host cell i.e the interactions depend on the formation of multicellular conjugates. This formalism is quite general, allowing in a simple way the mathematical representation of different detailed mechanism for the cell interaction, all of them dependent on the formation of multicellular conjugates. Mathematically the inherent difficulty of the latter problem resides in counting the multicellular conjugates classified according their stoichiometry, since minimally one differential equation is needed for every possible class of conjugate, increasing substantially the total number of equations in the model. The advantage of our approach here relies on the derivation, under a reasonable set of biological approximations, of an analytic formula for the number of different conjugates. This formula is rather complicated, but still tractable and considerably advantageous for the analysis of the model. Our formalism may very well be used to study different biological problems besides the study of T cell mediated suppression phenomena carry out here. The obvious example in this sense is the study of the interaction between the helper and the cytotoxic T cells following the model proposed by Mitchitson et al [7], which more than analogous is roughly identical to the problem addressed here (see discussion in section 2.1). Moreover our formalism can be further generalized outside the framework of problems in cell population dynamic. Note that the role of the host cell in the model is just to co-localize the cells in the interaction, therefore the formalism represents also a model for the dynamic of several populations of individuals whose interaction depend in their spatial co-localization in a given set of specified locations. Examples of biological problems that could profit of our mathematical formalism by the latter analogy can be easily conceived. For instance, consider some problems of modeling the regulations of genes

transcription in a cell. Gene promoters are typically made of repetitive transcription elements to which several transcription factors can bind. The transcriptional activity is often dependent on the precise stoichiometry of the transcription factor assembly that will contain different subunit activator or repressor factors. Many times the same DNA sequence can be a binding element for different transcription factors/subunits. The formalism for describing the stoichiometry of multicellular conjugates derived in this thesis could very well be used to model these situations. A completely different example can be envisaged in problems of population dynamic in ecology. Imagine a system of predator and prey where the predation is efficient only while the prey is drinking water in the margin of a river. In this case the interaction between the species only take place if they are close enough to each other along the line of the river margin when the prey come to drink. A simple strategy to model this problem could be to subdivide the river margin in equivalents sections where the predation can take place, using the formalism derived in this thesis to compute the probability that a predator and the prey are co-localized in a given margin section at a given time. Many other examples of analogous problem could be drawn down here. But the bottom line is that our formalism can be potentially useful in the resolution of several analogous biological problems and we hope that it proves useful to other modelers.

Despite the potential value referred above, there are some limitations on the application of the mathematical formalism developed in this Thesis. There are some technical difficulties in applying the formula for the number of multicellular conjugates provided in this Thesis. On the one hand this formula use discrete numbers of T cells and APCs in the calculation. This fact leads to discretization problems, which should be handle carefully in each particular case. In our studies here we avoid this problematic by working with large values of the number of T cells and APCs, but this may not be possible in other applications. On the other hand, this formula is combinatorial in nature. This fact makes it computationally heavy and demands some strategies of tabulation to viabilaze its practical application. Moreover there are some limitations of the formalism, which are biological rather than technical. The possible cooperativity effects on the process of conjugation and de-conjugation of T cells to the muslticellular conjugates have been neglected in our studies. This assumption is taken as a first approximation to the complex issue of mathematically describing the process of cognate intercellular cooperation, although we are aware that either positive or negative cooperativity potentially could play a role in this process since there is a large number of different molecules that participate on it. In practical terms cooperativity means that the conjugation affinity of the T cells for an APC, which is free of other T cells, might be different from the conjugation affinity for an APC, which is already bound to other T cells; or in the context of the more general

formulation of our formalism provided above, that an individual might have a different probability to get localized in given place depending in the presence of other individual in the same place. Our formalism cannot be applied to those problems where cooperativity is significant. Further development of the formalism is required to access this issue.

3.3.2 Towards a taxonomical approach of modeling

Another interesting aspect of the application of modeling methodologies in this Thesis is the use of the mathematical models for hypotheses comparison and testing performed in our chapters 4 and 5. In those chapters a set of alternative models were set up for a particular biological problem, comparing the biological hypotheses they represent by their capacity to explain available experimental data. The latter strategy of modeling was quite successful. We formulate 4 candidate models, representing different mechanistic hypotheses given in the literature, finally reducing the number of acceptable candidates to only two mechanisms and revealing in the mean time some interesting aspects of the phenomenon studied. In our experience this strategy for modeling render the theoretical analysis quite suitable for the experimentalist. Perhaps due to the fact that one starts the modeling by representing in mathematical terms the hypotheses that were conceived by the experiments, adding to the analysis a quantitative view that is most typically lacking there.

The studies in this Thesis encourage us to put forward here a novel strategy for conducting mathematical modeling in some biological problems. We refer to this strategy as the "taxonomical approach" for modeling and the idea derives from an analogy with the building of taxonomical tree of species in biology. In a taxonomical tree, the properties of the different families and subfamilies of a kingdom are specified so that using this information a newfound specimen can be easily classify in a given family and subfamily. We know that birds have feathers and mammals have mammalians glands; therefore just by looking to those properties a new specimen can be classified. Our proposal is to build taxonomical tree for models of alternative hypotheses of a given biological problem where those critical properties that allow distinguishing the models and consequently the alternative hypotheses are specified. Building such a taxonomical tree of models would have advantages both for the experimentalist and the theoreticians, forcing in some way their mutual interaction. On the one hand, for the experimentalist the tree would provide a guide for the experimentation of the given problem. Note that although some of the experiments to distinguish alternative hypotheses may be intuitively obvious for the experimentalist, others may not be so and therefore the use of the mathematical model would be most useful for designing new experiments. In this sense the model may help to clarify the formulation of every alternative hypotheses and to

derive their most ultimate, quantitative and unexpected consequences. On the other hand, for the theoreticians, building the tree would help to systematize and organize the knowledge of several mathematical models around a general and useful theoretical framework that could represent a starting point for their systematic application and extension to other analogous problems. The taxonomical approach for modeling might be especially useful if applied to a problem sufficiently general so that the tree generated can be used to address many particular biological problems. This poses another interesting and difficult task to the theoreticians, the one of formulating the models in the most simple and general form possible.

But let us exemplify in a particular situation this taxonomical approach for modeling. Consider the problem of identifying the mechanism of interaction between two cells population. This problem is extremely general; since it could be specifically applied to any two-cell populations of interest. Therefore having a taxonomical tree of this will provide a clear recipe for the experimental characterization of the mechanism of interaction between any two cells of interest. A particular case of the latter problem is the study of the mechanism for the interaction between the E and R cells in T cells

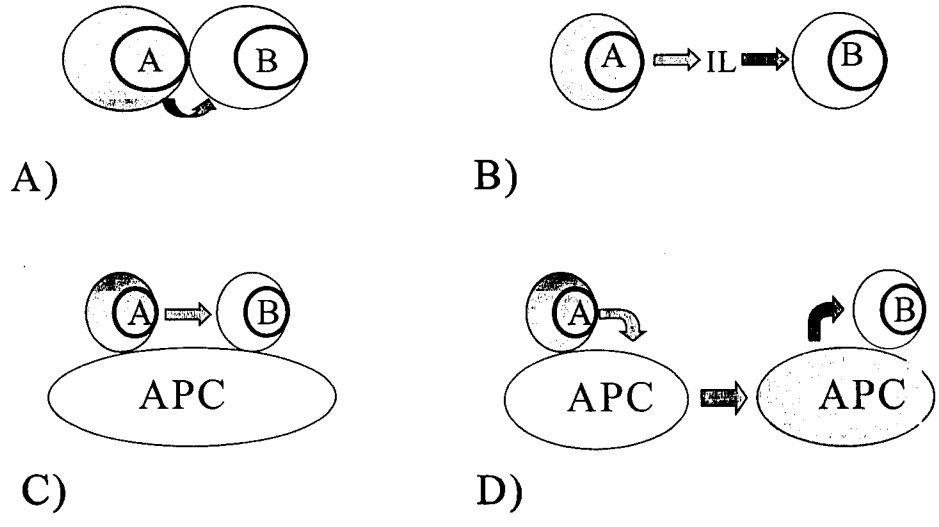

Figure 3. Alternative mechanisms for the interaction between any pair of cells, A and B in a biological system.

mediated suppression addressed in the section 2.1 to 6 of this thesis. Despite the generality of the problem stated, in theory there are just 4 main mechanisms by which such interaction can occur in nature (figure 3). A) The interaction can be mediated by a direct cell to cell interaction; B) The interaction can be mediated by a soluble factor secreted to the medium; C) The interactions can be

mediated by the simultaneous conjugation to a third host cells (like in T cell mediated suppression); And D) the interaction can be mediated by a third host cell, but through its asynchronous conjugation with the cells of interest and not in a synchronous conjugation as in the mechanism C. For each one of these major hypothetical mechanisms a mathematical model can be built and those predictions of the models corresponding to testable experiments, which distinguish among alternative hypothesis, can be identified being added to the taxonomical tree. Longer branches of the tree can be further added by modeling more detailed aspect of the interaction between the cells, for instance following the candidate hypotheses modeled in section 2.1, which are all compatible with the mechanism C for the interaction between the R and the E cells in T cells mediated suppression. Interestingly in the problem exemplified here, like perhaps in many others that could be approached in this way, more than building new models for the tree we just need to recompile and compare many models that have been previously studied in the literature. For instance models of class A can be found in the models of idiotypic network theories [50, 51] or those of T cells vaccination phenomena [52, 53]; models of class B can be found in the work Fishman et al. [54] and Carneiro et al [55]; And models of class C are provided for the first time in the present Thesis. Note that just compiling these models in a simple and general theoretical framework will add considerable value to the previous theoretical work.

3.3.3 But why mathematical models in biology and in this Thesis?

Finally after going through the experience of this Thesis, it is worth given a try to answer the question above, which is typically asked to modelers by most biologist. A first obvious reason is that the author received basic training in physics and like many other physicists and mathematicians became interested in biological problems. We of course would like to apply our skills, and previous knowledge to these biological problems. But of course this is neither the only nor the major reason.

In my opinion the main reason to use mathematical models in biology is the fact that they are good tools to establish a bridge between different levels of organization, and biological systems are typically hierarchical system with many relevant levels of organization. But let's elaborate this idea in the context of the cell population dynamics models in this Thesis. In these models several postulates, assumptions about how individual cells interact to each other are included. For instance, a postulate could be Regulatory and Target T cells interaction require linked recognition at the APC surface or another postulate could be Target T cells produce a cytokine that serve as growth factor to the regulatory T cells. Note that these are assumptions on how two individual cells interact to

each other. But once resolved the model, several problems related to how a given population of cells behave could be addressed. For instance, we could ask whether the population of regulatory and target T cells can coexist on equilibrium? Or whether there exist a global state dominated either by regulator or Target T cells? So in this way the model is establishing a link, a bridge between the process that take place at the cellular level and the phenomenology observed at the level of cell populations. More precisely the model says how a specific type of cell interaction is reflected or contributes to the behavior of the cell populations. Note that this is precisely the problem of biology in many different levels. We are interested in understanding, for instance how the cells behave in term of the interaction of the gene network that composes it? or How an ecological system evolve from the characteristics and habits of the different species on it? Moreover in some branch of Biology, like in Immunology, there is a considerable amount of information about different cellular processes that occur *in vitro* or under certain particular conditions. But there is no answer to the obvious question of whether they may be relevant in the physiology of the system. This latter type of problem is quantitative in nature, therefore in our opinion it will require mathematical modeling for its final resolution.

But of course you can approach these biological problems without using mathematical models and most biologist have done and do so. Nevertheless the complexity of these systems, given by the facts that they are composed of many elements interacting with each other and that often these interactions are non-linear, suggest mathematical modeling could be an adequate technique. Moreover, in some cases the actual interaction in the lower organization levels is technically inaccessible or it is still not fully understood, so the process at this levels need to be inferred from the observation at the higher level of organization. This type of problem is again quite suitable to mathematical model and it is pretty common in statistical physics. An example of the latter is observed in the problem addressed in this Thesis. The actual interaction between targets and regulatory T cells is not completely understood and it would be difficult to directly access it at the single cell level. Moreover even if you manage to do so in some extremely artificial condition, one will further need to study what's the contribution of the observed phenomena in the overall behavior of the immune system.

3.4 References

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