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RESPOSTA IMUNE A PEPTÍDEOS DERIVADOS DA IMUNOGLOBULINA: DA TOLERÂNCIA À AUTO-IMUNIDADE

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Resumo

A interação de uma célula B com uma célula T ativada na borda T-B leva a formação de um centro germinativo que tem como resultado o aumento da especificidade e afinidade da célula B pelo antígeno. Após a formação de uma célula B de alta especificidade e afinidade, alguns clones diferenciam-se em células produtoras de anticorpos. O anticorpo é a molécula efetora da célula B e tem como principais finalidades a opsonização, a neutralização, a fixação de complemento e a imunomodulação. Na maioria dos indivíduos, a resposta imune mediada por anticorpo é direcionada apenas contra antígenos não-próprios, porém, devido a fatores até hoje não compreendidos, alguns indivíduos montam respostas auto-imunes. Pouco se sabe quais os mecanismos que estão por trás da formação e manutenção das células auto-reativas. Em um dos modelos que propõe como as células B são recrutadas em resposta auto-imunes, como no Lupus Eritematoso Sistêmico, as células B recebem auxílio via apresentação de peptídeos derivados do receptor de célula B. Os linfócitos B podem aleatoriamente combinar diversos segmentos gênicos para criar um único receptor na superfície da célula. Devido à baixa freqüência de uma célula B expressar um gene V em particular, originalmente foi proposto que as células T não eram tolerantes aos peptídeos derivados da imunoglobulina. Em virtude que a auto-imunidade não é a regra, mecanismos que controlam a resposta de células T a peptídeos derivados da imunoglobulina se fazem necessários. Este trabalho tem como objetivo testar estas duas hipóteses. A primeira questão é a de como as células T imunoglobulinas específicas são tornadas tolerantes. A segunda questão é a de que uma célula B auto-reativa pode ser conduzida a uma resposta auto-imune pela apresentação de peptídeos derivados da imunoglobulina. Neste modelo, apenas as seqüências da imunoglobulina que sofressem hipermutações somáticas teriam o potencial de gerar um epitopo de célula T. No segundo capítulo, usando uma combinação de dois animais transgênicos e a técnica de transplante de medula óssea, um terceiro animal transgênico foi gerado. Neste modelo, a freqüência de uma célula B expressando um gene V em particular (células B 3671-κTg) é semelhante à diversidade do repertório de células B, e as células T(CA30) são, na grande maioria, específicas para um peptídeo derivado da cadeia κ das células B 3671-κTg. Os resultados demonstraram que as células T são, na grande maioria, eliminadas no timo destes animais, porém algumas células são capazes de alcançar a periferia. As células que escapam o timo aparentemente expressam uma cadeia α endógena, são antígenos experientes, porém não respondem à estimulação in vitro com o peptídeo. O terceiro capítulo teve como objetivo testar a apresentação do receptor como via para as células B auto-reativas, usando um animal transgênico parcial para o receptor de célula B. Apesar de a auto-imunidade ter sido induzida de forma espontânea nesses animais, nenhuma célula B auto-reativa encontrada na periferia expressava o receptor transgênico.

Palavras-Chave: SLE; auto-anticorpos; tolerância; células T; peptídeos derivados imunoglobulina.

Summary

The interaction between a B cell and a primed T cell , in the T-B border, leads to a germinal center reaction, where an antigen specific B cell increases the affinity and specificity for the antigen. After the generation of the high affinity and specificity, some clones differentiate into antibody producing cells. The antibody is the effector molecule of the B cell, and has major functions such as opsonization, neutralization, fixing complement and immunomodulation. In most individuals, the antibody mediated immune response is exclusively specific to non-self antigens, but for reasons not well understood, some individuals develop autoimmune responses. Little is known about the mechanisms behind the generation and survival of autoreactive cells. One of the models of how B cells are recruited in autoimmune responses, like Systemic Lupus Erythemathosus, predicts that B cells are driven into an autoimmune reaction through the presentation of peptides derived from the B cell receptor. The B lymphocytes can randomly combine several gene segments, generating a single cell receptor. Because the frequency of a B cell expressing a particular V gene is low, it was originally postulated that T cells were not rendered tolerant to immunoglobulin derived peptides. Since autoimmunity is the exception, not the rule, mechanisms that suppress the T cell responses against immunoglobulin derived peptides are required. The goal of my Thesis is to test two hypotheses. The first is how immunoglobulin specific T cells are rendered tolerant. The second is to test if a B cell can be recruited in an autoimmune response, through the presentation of immunoglobulin derived peptides. In this model only the sequences of the immunoglobulin that have somatic hypermutated have the potential to generate a T cell epitope. In the first chapter, using a combination of two transgenes and the mixed bone marrow technique, a third animal was generated. In this model the frequency of a B cell expressing a particular V gene (3671-κTg B cells) is similar to the B cell repertory diversity, and the T cells (CA30), are specific to peptides derived from the κ-chain of 3671-κTg B cells. The results demonstrated that the majority of the CA30 T cells were deleted in the thymus; however some T cells were able to reach the periphery. The escapees expressed an endogenous α-chain, were antigen experienced, but did not proliferate to peptide stimulation in vitro. The second chapter's objective was to test the receptor presentation hypothesis, as an avenue for T cell help to autoreactive B cells, using a partial transgene animal to the B cell receptor. Even with autoimmunity being spontaneously induced, no autoreactive B cell carrying the transgene autoreactive receptor was found in the periphery.

Keywords: SLE; autoantibodies; tolerence; T cells; peptides derived from immunoglobulin.

CAPÍTULO I

1.1 Introdução Geral

1.1.1 O Sistema Imune Adaptativo

Uma das grandes conquistas evolutivas dos animais vertebrados foi o desenvolvimento de uma imunidade adaptativa com células especializadas (linfócitos) capazes de gerar uma resposta protetora e específica a quase qualquer forma de agente agressor.

A capacidade dos linfócitos em gerar um leque tão amplo de respostas contra os mais diversos antígenos - e aqui se inclui desde toxinas até tumores - é diretamente proporcional à habilidade de combinar algumas centenas segmentos gênicos da família das imunoglobulinas (Ig) de maneira aleatória. A recombinação gênica, a adição de nucleotídeos e as junções imperfeitas contribuem juntas para formação dos receptores de células T (TCR) e B (BCR) que são quase únicos entre uma população de linfócitos, como uma espécie de impressão digital dos mesmos. Tamanha diversidade só se torna útil na defesa contra agentes patogênicos quando os diversos clones de linfócitos interagem entre si. Através dessa rede de comunicação, clones de linfócitos mais específicos são selecionados no decorrer de uma resposta imune, em um processo que se assemelha à teoria evolutiva de Charles Darwin, em que os clones mais aptos se multiplicam exponencialmente, o que, na maioria dos casos, culmina com o silenciamento do antígeno.

1.1.2 O Linfócito B

Os linfócitos B são células especializadas, cuja principal função é a produção de anticorpos. O anticorpo nada mais é que a forma solúvel do BCR que tem como funções a opsonização, a neutralização, a fixação de complemento e a modulação da resposta

imune(1). Para um progenitor de linfócito B se diferenciar em uma célula secretora de anticorpo, primeiro ela precisa produzir um BCR funcional e não auto-reativo(2, 3).

Durante o desenvolvimento de uma célula B na medula óssea (BM), um segmento V da cadeia pesada (VH) recombina-se com os de diversidade (D) e de junção (JH) dessa mesma cadeia, gerando a cadeia pesada da Ig (VHDJH). Aproximadamente pelo quinto dia de vida do indivíduo, o gene da TdT torna-se ativo, o que aumenta a diversidade do repertório, pois através da TdT nucleotídeos aleatórios podem ser adicionados à cadeia pesada. A TdT atua na junção do VH com o segmento DJH, na Região Determinadora de Complementaridade 3 (CDR3), uma das regiões responsáveis pela ligação antígeno-anticorpo(4). Uma vez montada uma cadeia pesada funcional em um dos alelos, o outro alelo é silenciado, em um processo chamado de Exclusão Alélica(5). Quando a cadeia pesada é expressa na superfície do progenitor da célula B, o *locus* da cadeia leve se torna acessível, a cadeia leve κ é a primeira a recombinar e, em caso de um rearranjo não produtivo, a recombinação ocorre na cadeia leve λ(6-9). Ao contrário da cadeia pesada, a cadeia leve não apresenta elementos D e não existe a adição de nucleotídeos pela TdT, o que limita a diversidade da cadeia leve em relação à cadeia pesada.

1.1.3 Controle das Células B Auto-reativas

Uma das mais extraordinárias habilidades do sistema imune é a capacidade de diferenciar o próprio do não-próprio. Através dela, a resposta imune é capaz de proteger contra os mais diversos agentes patológicos sem gerar danos ao organismo.

A recombinação de vários segmentos gênicos é capaz de gerar clones de linfócitos B com as mais diversas especificidades, em que linfócitos com BCR contra antígenos próprios e não-próprios são criados. Com a finalidade de evitar que as células B auto-

reativas participem em uma resposta auto-imune, as células B são submetidas a vários "checkpoints" de tolerância(10, 11). Um dos mecanismos pelos quais as células B são silenciadas, durante o estágio imaturo, é chamado Edição do Receptor(6,9). Nesse processo, quando há formação de um BCR auto-reativo na medula óssea, as RAG(12) são expressas induzindo um novo rearranjo no mesmo alelo da cadeia leve κ. Se o BCR mantém-se auto-reativo, o segundo alelo da cadeia leve κ é rearranjado. Quando esgotadas as tentativas na cadeia κ, a cadeia leve λ sofre o rearranjo gênico. Quando há substituição de um BCR auto-reativo por um não auto-reativo, o linfócito B deixa a medula óssea e entra na circulação. Caso a edição do receptor não elimine o potencial auto-reativo do BCR, este linfócito B entra em um processo de apoptose, em um mecanismo chamado de Deleção Clonal. Nem todas as células B auto-reativas são eliminadas por Edição ou Deleção na BM. Aproximadamente 6% do repertório de células B na periferia são formados por células B anérgicas(13). A anergia acontece quando há o encontro de uma célula B auto-reativa com um antígeno próprio no organismo, na ausência de uma célula T antígeno específica(14). Não se sabe ao certo o que determina o destino de uma célula B durante o desenvolvimento na BM, mas sabe-se que o sinal emitido pela ligação antígeno-BCR exerce uma grande influência. Um quarto mecanismo pelo qual as células B auto-reativas são silenciadas consiste na diferenciação dos linfócitos B em células não foliculares como, por exemplo, células de Zona Marginal (MZ) no baço, ou células B do subtipo B1 na cavidade peritoneal(15, 16). Esses linfócitos B normalmente são a primeira linha de defesa da imunidade adaptativa, gerando uma resposta de anticorpos aos primeiros dias de exposição ao patógeno(17). As células B não foliculares dificilmente participam em uma resposta T dependente que é caracterizada pelo processo de hipermutação somática do BCR.(18). Outra característica da resposta de anticorpos gerada pelas MZ ou B1 é que estas células dificilmente sofrem o processo de troca de

classe, e isso é de fundamental importância, já que auto-anticorpos da classe μ (IgM) raramente são patogênicos(19).

1.1.4 O Linfócito T

Semelhante ao processo de recombinação das células B, o linfócito T recombina segmentos gênicos para formar o TCR, um processo que, ao contrário das células B, acontece no timo. As células T CD4 ou CD8 recombinam as cadeias α e β , que correspondem estruturalmente à cadeia leve e pesada do BCR.

Ao contrário dos linfócitos B, as células T reconhecem apenas antígenos peptídicos no contexto de uma molécula de MHC. As células T CD4 reconhecem peptídeos apresentados no contexto de MHC classe II, e as células TCD8, em um contexto de MHC classe I. O processo de diferenciação do linfócito T em célula T CD4 ou T CD8 acontece no estágio duplo positivo (CD4+CD8+) em que uma célula T é capaz de receber um sinal positivo de um dos dois tipos de MHC. Este estímulo faz com a célula T diferencie-se em uma célula CD4, em caso de um sinal positivo do MHC classe II, ou CD8, em caso de um sinal positivo do MHC de classe I. Células T que não reconhecem nenhum dos dois tipos de MHC morrem por apoptose, em um processo chamado Morte por Negligência. Um recente modelo propõe que as células T duplo positivas sejam induzidas a morrer por um processo induzido pela molécula CD8(20). O processo pelo qual uma célula T define a especificidade pelo MHC é chamado de Seleção Positiva(21).

1.1.5 Os Linfócitos T e a Seleção Negativa

Como nos linfócitos B, o processo de rearranjo gênico combina aleatoriamente segmentos gênicos que, por sua vez, podem gerar TCR auto-reativos ou não. Para evitar que células T com potencial auto-imune participem de respostas imunes na periferia, elas são submetidas a um processo chamado de Seleção Negativa(22). A Seleção Negativa

consiste na apresentação de antígenos próprios no contexto de moléculas de MHC de classe I ou II, em que células T que recebem um sinal forte do complexo peptídeo-MHC são eliminadas. Estima-se que a maior parte das células T é eliminada neste estágio, e apenas uma pequena parcela das células torna-se células T maduras, podendo assim alcançar a periferia.

1.1.6 Expressão Ectópica e Genes AIRE

O processo de Seleção Negativa tem a função de modelar o repertório de células T, eliminando as células T auto-reativas. Porém, a diversidade de antígenos próprios presentes no nosso organismo, muitos dos quais expressos em tecidos não-linfóides, oferece um desafio ao sistema imune. Uma maneira encontrada pelo sistema imune de controlar a existência das células T auto-reativas é através da expressão ectópica de proteínas que são tecidos específicos. A expressão ectópica é controlada pelos chamados genes AIRE, e indivíduos que apresentam defeitos nesses genes sofrem manifestações precoces e graves de doenças auto-imunes(23).

1.1.7 As Células T Regulatórias

Apesar de o processo de Seleção Negativa eliminar a grande maioria dos linfócitos no Timo, é difícil acreditar que as células T tornam-se tolerantes contra todos os antígenos próprios possíveis. Para assegurar que respostas auto-imunes não ocorram, um tipo especial de linfócito T foi evolutivamente selecionado. As células T regulatórias naturais (Treg) são linfócitos T com receptores auto-reativos que, ao invés de sofrerem apoptose, se diferenciam em uma população especial de linfócitos que possuem a habilidade de suprimir as respostas imunes. O que determina se uma célula T auto-reativa morre ou diferencia-se em Treg é ainda incerto, mas acredita-se que a intensidade do

sinal recebido pelo TCR é um fator importante nesta escolha, em que células com alta afinidade por antígenos próprios diferenciam-se em Treg(24,26).

1.1.8 Célula T Ativada e Anergia

Uma célula T que deixa o Timo e entra na circulação, com direção aos órgãos linfóides secundários, é chamada de célula T virgem. Uma célula T virgem só pode participar de uma resposta imune se antes for ativada por uma célula apresentadora de antígeno (APC) que, na maioria das vezes, é uma célula dendrítica (DC). A ativação da célula T acontece nos órgãos linfóides secundários em que uma APC captura um antígeno, realiza o processamento para só então apresentar um peptídeo derivado deste antígeno no contexto de uma molécula de MHC de classe I ou II. Este reconhecimento do complexo peptídeo-MHC pelo TCR é conhecido como sinal 1. Uma célula T virgem só se torna uma célula T ativada se, quando da presença do sinal 1, houver um sinal 2. O sinal 2 é normalmente chamado de co-estimulação, e o tipo de co-estimulação mais estudada é o da interação CD28 (célula T) - B7 (APC). A presença de moléculas co-estimulatórias na APC, está relacionada com a presença ou não de mediadores inflamatórios. Exemplos clássicos de mediadores inflamatórios são os agonistas de receptores tipo TOLL (TLRs). O reconhecimento de antígenos, associado com um sinal via TLRs nas APCs, gera uma cascata de sinalização que culmina com a apresentação de antígenos no contexto de uma molécula de MHC mais moléculas co-estimulatórias(27,29).

Caso a interação de uma célula T com o complexo peptídeo-MHC aconteça sem a presença de co-estimulação, sinal 1 sem sinal 2, a célula T virgem torna-se uma célula T anérgica(30) em que esta se torna refratária a futuras estimulações. O processo de anergia é um exemplo dos vários mecanismos pelos quais o sistema imune controla as respostas auto-imunes.

1.1.9 Tolerância, Auto-Imunidade e Lupus

O sistema imune tem como meta gerar uma resposta protetora a um agente patogênico, sem que para isso mais mal seja feito. Tolerância é o fenômeno pelo qual os linfócitos auto-reativos são silenciados. Entretanto, existem incontáveis exemplos em que o sistema imune, por razões que na maioria das vezes são incompreendidas, passa a reconhecer antígenos próprios como agentes potencialmente prejudiciais ao organismo, gerando uma resposta auto-imune.

Um dos exemplos mais estudados de doença auto-imune é o Lupus Eritematoso Sistêmico (SLE), uma doença que é caracterizada pela presença de auto-anticorpos contra estruturas nucleares, principalmente DNA(31).

Pouco se sabe a respeito dos mecanismos que levam à quebra da tolerância no SLE, mas acredita-se que, para o desenvolvimento de auto-anticorpos, tanto células B e T auto-reativas são necessárias.

Várias linhas de evidência sugerem que a resposta mediada por auto-anticorpos segue o modelo clássico de uma resposta T antígeno-dependente, onde os anticorpos apresentam diversas hipermutações somáticas e Ig da classe γ (IgG). Em acordo com esse modelo, o bloqueio da interação T-B leva, a uma atenuação das manifestações do SLE

1.1.10 Células B Auto-Reativas na Periferia

Apesar dos inúmeros “checkpoints“ de tolerância que dão forma ao repertório de células B, um grande número de relatos sugere que células B auto-reativas estão presentes na periferia.

Experimentos realizados, através de imunizações de linhagens de camundongos com mimetopos de DNA, induzem uma resposta humoral mediada por auto-anticorpos

anti-DNA(32, 33). Outra publicação do mesmo grupo mostra que algumas linhagens são mais susceptíveis que outras na indução de auto-anticorpos, e os autores sugerem que isso é conseqüência direta da intensidade do sinal do BCR durante o desenvolvimento da célula B(34). Na mesma corrente de pensamento, experimentos em que a transferência de células T capazes de interagir com uma célula B de uma maneira que seja independente do antígeno reconhecido pelo BCR, têm como conseqüência a produção de auto-anticorpos anti-DNA(35-37).

1.1.11 Origem dos Anticorpos Anti-DNA

Embora a maioria dos pesquisadores acredite na existência de células B auto-reativas na periferia, a origem dos anticorpos anti-DNA é controversa. Usando BCR transgênicos (Tg) para antígenos nucleares, em que virtualmente quase todas as células B inicialmente expressam um BCR auto-reativo, elas não são capazes de induzir uma resposta de auto-anticorpos(38, 39), pois a maioria das células B auto-reativas foi silenciada quando da presença de um BCR auto-reativo, isto em linhagens de “background” genético não auto-imune. Quando estes Tg foram retro cruzados em linhagens auto-imunes, a presença de um BCR auto-reativo apenas modestamente acelera as manifestações do SLE.

Uma interpretação desses resultados é que uma célula B para ser recrutada em uma resposta auto-imune precisa adquirir um potencial auto-reativo numa fase mais tardia do desenvolvimento. Através da clonagem de IgGs anti-DNA, Winkler e colegas demonstraram que, no momento da reversão das hipermutações somáticas para forma germline do anticorpo, a especificidade do auto-anticorpo por DNA era eliminada(40).

1.1.12 Células T Auto-Reativas e SLE

Diferentemente dos linfócitos B auto-reativos, a especificidade das células T, no Lupus, é controversa. Existem principalmente três modelos que tentam explicar a origem deste auxílio.

O primeiro modelo, e o mais difundido, é o que afirma que as células T são específicas contra peptídeos derivados das histonas. Estudos realizados por diversos grupos, usando seqüências peptídicas derivadas das histonas, foram identificadas células T histonas específicas. Um outro trabalho publicado, utilizando clones de células T auto-reativas, demonstrou que as células T auto-reativas tinham a tendência a resíduos negativamente carregados nas cadeias α do TCR(41-43). Entretanto, apesar de este modelo estar presente há mais de duas décadas, nenhum estudo realizado usando animais Tg pro TCR anti-histona foi feito até hoje.

Um segundo modelo propõe que as respostas auto-imunes no SLE iniciem como uma resposta a antígenos não-próprios e que, através de reações cruzadas, iniciem as respostas auto-imunes. Neste modelo, as células T auto-reativas reconhecem um antígeno não-próprio com alta afinidade, mas respondem a antígenos próprios em baixa afinidade (mimetismo molecular). A melhor evidência desse modelo foi apresentada em um estudo, usando pacientes juvenis portadores de SLE, em que aproximadamente 100% dos pacientes com SLE apresentaram infecção por Epestein Barr vírus contra cerca de 70% do grupo controle(44).

O terceiro modelo é o da apresentação do receptor em que as células B auto-reativas recebem auxilio via apresentação de peptídeos derivados do BCR. Neste modelo, a hipermutação somática no BCR gera um antígeno até então desconhecido pelas células T, o que, por sua vez, cria uma via para o auxílio antígeno independente das células B. Em favor deste modelo está a presença de células T específicas para peptídeos derivados da Ig no fluido cérebro-espinal em pacientes com Esclerose

Múltipla(45). Uma segunda linha de evidência é a da transferência de células T específicas para antígenos derivados do BCR. Nestes experimentos, as células B são recrutadas em uma resposta imune independente do antígeno, o que culmina com a produção de auto-anticorpos(36, 37).

1.1.13 Células T Ig Específicas

Experimentos em que células T Ig específicas foram injetadas em animais BCR Tg levaram à produção de auto-anticorpos pelas células B Tg, demonstrando que, se fornecida uma via de auxílio T antígeno irrelevante, as células B diferenciam-se em células auto-reativas. Entretanto, auto-imunidade não é uma regra, o que nos leva a acreditar que esta via é silenciada na maioria dos indivíduos.

Análises das respostas de células T a mAbs revelaram que as células T reconhecem peptídeos derivados de seqüências que sofrem mutações, mas não seqüências não-mutadas(46). Uma exceção a esta regra é a presença de células T específicas às junções VH-DJH em que, a adição de nucleotídeos aleatórios pela TdT, torna impossível a diferenciação entre mutação e diversidade juncional.

Experimentos usando camundongos transgênicos mostram que, na presença de altos níveis de um gene V específico, as células T Ig específicas são eliminadas no timo por Seleção Negativa(36, 47, 48). Apesar das evidências de seleção negativa, parece muito improvável que este seja o único mecanismo pelo qual as células T são tornadas tolerantes. A presença das diversidades juncionais e das hipermutações somáticas indica que mecanismos periféricos devem operar no sistema a fim de evitar o auxílio antígeno independente, entretanto nenhuma evidência destes mecanismos foi publicada até hoje na literatura.

1.2 Hipóteses e Problema

O controle das respostas T a antígenos derivados da Ig é uma forma de o sistema imune evitar respostas auto-imunes. Várias evidências sugerem que o sistema imune, em condições normais, é tolerante aos peptídeos derivados da Ig, entretanto nenhum estudo detalhado demonstrou, em situações fisiológicas, quais os mecanismos que controlam as respostas T a estes peptídeos. Da mesma forma que são raros os estudos demonstrando os mecanismos pelos quais as células T são tornadas tolerantes, nenhum estudo até hoje demonstrou claramente qual a especificidade das células T auto-reativas no SLE. Existem vários modelos que sugerem uma ou outra teoria, mas todos os modelos até então apresentados possuem pontos fortes e fracos.

Esta tese tem como objetivo principal testar essas duas perguntas. A primeira é de como o repertório de células T é tornado tolerante aos peptídeos derivados da Ig. A segunda é buscar evidências de que as células B auto-reativas são recrutadas via apresentação de peptídeos derivados do BCR somaticamente hipermutados.

Para identificar os mecanismos que governam a tolerância T a peptídeos derivados da Ig, foi utilizado um sistema previamente descrito com dois animais Tg. O primeiro foi um animal BCR Tg (3671-κTg); nele virtualmente todas as células B expressam a cadeia κ Tg. O segundo animal foi um TCR Tg (CA30) que reconhece um peptídeo derivado da cadeia κ das células B 3671-κTg no contexto de IA-k, através da geração de quimeras de medula óssea, em que a freqüência das células B Tg é semelhante ao repertório B normal. Através deste modelo foram testadas se as células T são ou não tolerantes a peptídeos derivados da Ig. Em caso de tolerância, esta acontece de uma maneira central, por seleção negativa ou periférica através da geração de células anérgicas ou regulatórias.

O segundo objetivo da Tese é a de criar um modelo experimental para testar se as células T Ig específicas podem recrutar células B em uma resposta auto-imune,

semelhante ao SLE. Para testar isto, um camundongo Tg parcial (pTg) para o BCR auto-reativo foi criado. Este pTg apresenta uma cadeia pesada que codifica para um BCR anti-cromatina, chamado pTg18R. Este auto-anticorpo é muito semelhante ao auto-anticorpo 18eh, com a exceção de dois aminoácidos na FR1 da cadeia pesada. Estes dois aminoácidos não interferem na afinidade nem na especificidade do auto-anticorpo por antígenos nucleares, mas criam um epitopo para as células T no contexto de IA-q. De uma maneira simplista, 18eh é a versão com o epitopo para as células T, e a 18R é a versão revertida deste anticorpo, isto é, sem epitopos para as células T no contexto de IA-q. O objetivo do trabalho era o de recrutar as células B carregando a cadeia pesada 18R em uma resposta auto-imune e o de testar se as células B auto-reativas adquiriram por mutações somáticas o epitopo T na região da FR1. Em caso positivo, isto indicaria que as células B auto-reativas são recrutadas em respostas auto-imunes via receptor.

1.3 Objetivos Gerais

1.3.1Testar se as células T são tolerantes a peptídeos derivados da Imunoglobulina.

1.3.1.1Desenvolver um modelo para o estudo da tolerância das células T a peptídeos derivados da Imunoglobulina.

1.3.1.2 Se tolerância for constatada, identificar os mecanismos pelos quais as células T são tolerantes a peptídeos derivados da imunoglobulina.

1.3.2Desenvolver um modelo para o estudo da apresentação do receptor da célula B como via de auxílio para as células B auto-reativas no Lupus Eritematoso Sistêmico.

CAPÍTULO II:

T cell Tolerance to Vκ Immunoglobulin Derived Peptide

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Keywords: Tolerance, T cells, Vκ peptides, Immunoglobulin

Abbreviations: **Tg**: transgenie; **Ig**: Immunoglobulin

Abstract

Our laboratory has shown that T cells are rendered tolerant to germline-encoded Ig V region-derived peptides, and that tolerance can occur by clonal deletion of self-reactive thymocytes. Thymic deletion is seen in "monoclonal mice", where unphysiologically high levels of a transgene-encoded Ig is present and where virtually all B cells express the same V gene.

The goal of this study was to determine how T cells are rendered tolerant to a germline Ig V region-derived peptide, under conditions in which the V region is expressed at physiological levels by small numbers of B cells. To this end, we created mixed bone marrow chimeras using donor bone marrow from mice expressing an MHCII-restricted Vκ-derived epitope and bone marrow from mice expressing an αβTCR specific for the Vκ-epitope (CA30). In chimeras where B cells expressing the Vκ-encoded peptide were present at low frequencies (0.8% to 6%), most of the CA30 cells were deleted in the thymus. However, a substantial fraction survived to the CD4 single positive stage, and some escaped to the periphery. Those in the periphery appeared to be tolerant as assessed in proliferation assays. On the basis of CD4 expression, the peripheral CA30 T cells could be divided into two groups, one of which showed clear signs of antigen experience and expressed FoxP3. These findings provide the first evidence that Ig V region-specific T cells are rendered tolerant through both central and peripheral mechanisms and that peripheral tolerance may involve regulatory T cells.

Introduction

The discovery that T cells can recognize immunoglobulin (Ig) derived peptides, providing antigen non-linked help to a B cell in the case of CD4+ T cells or suppression of an antibody response by a CD8+ T cell(1-8), raises an important question. How does the immune system generate an antigen specific antibody response without B cells receiving unwanted help or being deleted by the T cells? This question becomes even more critical if we take into consideration that the B cell receptor (BCR) is a byproduct of the recombination of several Ig genes, resulting in a protein that is almost unique among the B cell pool.

Studies done in this field have proposed that the T cell repertory is tolerant to the Ig germline genes, with the exception of CDR3 in the antibody region (8-15).Taking advantage of the Ig haplotype differences between several strains of mice, Majlessi et al demonstrated that Ig specific T cells are deleted in the Thymus in a B cell dependent process that correlates with Ig mRNA expression in the Thymus(15). Supporting this model, Snyder et al and Bogen et al, used a transgenic (Tg) Ig specific T cell receptor (TCR) to show that Tg Ig specific T cells were deleted in the Thymus(16, 17). The Tg T cell deletion was a process that did not require direct B cell presentation, but instead high levels of soluble Ig.

Although the previous studies provide a compelling body of evidence that T cells are rendered tolerant to Ig derived peptides, they fail to address the question of how T cells see the Ig V gene diversity and become tolerant. Because most of the experiments used high frequencies of a specific B cell, we created a system in which the frequency of a particular V gene was controlled to mimic a normal B cell repertory (around 100 VH genes for mice, assuming that there is no bias to a particular VH in mice, the frequency of a V gene would be 1:100). This was tested utilizing a complementary pair of Tg animals

developed in our lab (16). The first was a κ-chain Tg (3671κTg) animal, which expressed the light chain of the 36-71 monoclonal antibody (mAb). The second was an αβ TCR Tg mouse (CA30 Tg) that recognized a Vκ FR1 peptide from the 36-71 mAb in the context of IA-k. Using these two Tg mice, a mixed bone marrow chimera (BMC) was created in which both Tg lymphocytes could be followed, and the κTg-B cell frequency resembled the frequency of a particular V gene among the other B cells. The result of this combination demonstrated that the majority of CA30 Tg T cells were deleted in the Thymus, but some were capable of full maturation and reached the periphery. The CA30 Tg escapees were antigen experienced T cells that appeared to express two α-chains, but were rendered refractory to Ig-peptide stimulation *in vitro*.

Material and Methods:

Mice

The BCR Tg (3671-κTg) and TCR Tg (CA30) mice, in an A/j background, were previously described (16). For the mixed bone marrow chimera experiments, 3671-κTg was crossed to C57BL/6J generating the 3671κTg-B6AF1 (3671κTg-F1). To be able to detect the T cells, independent of the TCR expression, we breed the CA30 to the B6.PL mice, generating the (CA30xB6.PL) F1, known for now on as the $CA30^{Thy1.1}$ mice. All mice were breed in house

Mixed Bone Marrow Chimera

The (B6xA/J) F1 mice were used as recipient mice in the mixed BMC. The animals received were lethally irradiated and reconstituted i.v. with $2x10^6$ progenitor cells from the $CA30^{Thy1.1}$ and 3671κTg-F1 in the indicated ratios (figure 1).

Cell Purification:

Progenitor cells were isolated from BM of donor mice, using the negative selection kit from Stem Cell Technologies (StemCell Technologies, Vancouver, BC), following manufactures instructions. Lymph nodes and Spleen cells from 3 months reconstituded mixed BMC were used for T cell purification, using a negative selection kit from StemCells Technologies.

Flow Citometry

For conventional antibody stains, cells were incubated for 20 minutes with various antibodies in staining buffer, (PBS + 2% FCS + 0.01% Na azide), or T cell culture medium in flat bottom 96 well plates at a concentration of 10^7 cells/ml. Cells were stained for

expression of CD3(clone 145-2C11); CD4 (clone H129.19); CD8α (clone 53-6.7); Vβ8 (clone MR5-2); CD44 (clone IM7); CD62L (clone MEL-14); CD16/32 (clone 2.4G2); CD11b (clone M1/70); and B220 (clone RA3-6B2); CD5; Foxp3 (all from Pharmingen, San Diego, CA).

For staining, with I-A^k 36-71 tetramer, 30 µl of cells, (suspended in RPMI 10% fetal calf serum at $3x10^7$ cells/ml) were incubated with 120 ng of tetramer for 3 hours at 37^{o}C. After incubation, antibodies used in conjunction with tetramer staining were added directly to each well and the cells were moved to 4^{o}C for 20 minutes.

Purified anti-mouse CD16/32 (clone 2.4G2) was included to block Fcγ receptors unless FITC labeled anti-mouse CD16/32 was used to stain the cells.

mAb 1763

The mAb 1763 recognizes the specific the FR1 of the 3671 κ light chain, do not cross react with the 3665 κ light chain (unpublished data).This antibody allow us to follow B cells and sera Ig derived from the 3671-κTg B cells.

Total IgG and IgM

In order to detect total IgG, 96 well trays were coated overnight at 4^0 C with 5 µg/ml of Fc specific goat anti-mouse IgG (Sigma, St Louis MO). All plates were incubated with blocking buffer for 1-2 hours at 37^{o}C. Mouse sera were incubated at the a starting dilution of 1:10000 for 1 hour at 37^{o}C. IgG antibodies were detected with biotinylated goat anti-mouse κ (Southern Biotechnology, Birmingham AL) followed by europium labeled streptavidin, (Wallac, Turku Finland). Europium fluorescence at 615 nm was measured on a Wallac Victor^2 1420 multilabel counter using an excitation wavelength of 340 nm, (Wallac). The IgM assay was done in a similar wasy, but instead of an anti-IgG an goat anti-IgM was used.

1763 mAb Competition Assay

To detect and quantitate 3671-Ig in the sera, 3671-Ig antibody concentration was determined by competition assay performed in 96 well trays (Corning, New York NY), that were coated overnight at 4^0 C with a mixture of 1763 mAb (1.667 µg/ml, produced in house) and normal rat gamma globulin (3.33 µg/ml, Sigma, St Louis MO). Plates were blocked for 1-2 hours at 37^oC. Sera were incubated at the indicated concentrations and competed with a fixed concentration of biotinylated-3671 mAb, produced in house. Biotinylated antibodies were detected by europium labeled streptavidin, (Wallac, Turku Finland). Europium fluorescence at 615 nm was measured on a Wallac Victor^2 1420 multilabel counter using an excitation wavelength of 340 nm, (Wallac).

Analysis of 3671-κTg B cells in Histolological Sections

For histology, spleens were embedded in Tissue Tek O.C.T. compound, (Sakura Finetek, Torrance CA), and frozen in a dry-ice cooled bath of 2-methylbutane. Samples were stored at $–70^0C$ until cutting. Tissue was cut in 6 µm to 8 µm sections on a Leitz Cryostat, (Leitz GmbH and Co. KG, Oberkochen Germany). Sections were applied to slides, air dried, acetone fixed and stored at -70^0C. After thawing, sections were rehydrated with PBS, (15 minutes), followed by blocking with blocking stainning solution Spleens were stained with B220-PE and mAb 1763-biotin. Biotinylated antibodies were detected with streptavidin conjugated to APC (Sigma). Sections were photographed using a Nikon Diaphot microscope, (Nikon Inc. U.S.A.), coupled to a Photometrics CCD camera, (Photometrics, Huntington Beach CA) and analyzed using IPLab Spectrum version 3.1a, (Scanalytics, Fairfax VA).

T cell Proliferation Assays

Unless otherwise indicated T cells from mixed BMC were purified with the StemSep columns described above prior to stimulation *in vitro*. For proliferation assays, $2x10^5$ purified T cells were cultured with $2x10^5$ lightly irradiated (1100 rads) splenocytes as a source of APC from an unmanipulated mouse (200 µl final volume). Stimulator peptide Vκ 36-71 FR-1 peptide (3671-Ig) was added at 1µM, and the culture was incubated for 4–5 days at 37° C in 5% CO_2. In all cases tritium was added during the final 20 hours of culture and incorporation was detected with a Wallac Microbeta, using Beta-Scint scintillation fluid, (both from Wallac, Turku, Finland). In some experiments T cells were previously incubated with carboxy-fluorescein diacetate succinimidyl ester (CFSE) and cultured for 3-5 days in the presence of 3671-Ig peptide

Results

Bone Marrow Chimeras

In order to control the expression of the Ig peptides available during the T cell development, a mixed BMC was generated using progenitor donor cells from a B cell κ-light chain Tg mouse (3671κTg-F1) and an αβ TCR Tg mouse ($CA30^{Thy1.1}$). The CA30Thy1.1 T cells are specific to a peptide derived from the κ-chain of the 3671κTg-F1 mice in the context of IA-k MHC class II. The peptide recognized by the $CA30^{Thy1.1}$ Tg T cells, requires two somatic mutations in codons 7 and 8, amino acids that are not present in the Ig germline sequence. Figure 1 is an illustration of the experiment design, in which 3 types of mixed BMC were generated. The mixed BMC were classified according to the percentage of 3671κTg progenitor cells used to reconstitute the animal. A control group was generated, using only $CA30^{Thy1.1}$ progenitor cells. Table I summarizes the congenic markers used to track the lymphocytes in the recipient mice. The Tg T cells were followed by a Thy1.1 allele and also by their TCR specificity (tetramer IAk-3671). To be able to identify the Tg B cells, an antibody specific to the 3671 κ-light chain was generated, called 1763 mAb. This mAb antibody is specific to the somatic mutations in the FR1.

B cell Engraftment is Proportional to Levels of Progenitor Cells Injected

Figure 2 shows the reconstitution of the recipient mice in the B cell compartment. As expected, as the number of progenitors injected decreased, so did the number of transgenic mature B cells. The numbers in the gates indicate the percentage of 3671κTg-B cells in a B220+CD11b/CD3- gate. The 3671κTg-B cells were also detected in the Thymus, in κTg chimera. Figure 3 shows a summary of the percentage of 3671κTg-B cells in the different lymphoid organs and blood. The numbers used to construct the graph were

obtained by subtracting the percentage of 1763+B cells in the control animals from the percentage of 1763+ B cells in the κTg chimeras.

Secreted 3671-Ig and the Relationship with the Frequency of 3671κTg-B cells

It was previously described that 3671-Ig, can induce thymic deletion of CA30 T cells(16). In this system high levels of IgG were thought to be responsible for the negative selection of Ig specific T cells. To measure the levels of 3671κTg-Ig a competition assay was developed using the 1763 mAb. Figure 4A shows the concentration of 3671κTg-Ig in the sera of the control, κTg^{low} and κTg^{int} chimera mice. As predicted no 3671κTg-Ig was detected in sera of the control animals. The concentrations of 3671κTg-Ig measured in sera of the κTg chimeras were proportional to the percentage of 3671κTg-Bcells, whereas the κTg^{int} had 2 times more 3671κTg-Ig when compared to κTg^{low}. It is interesting to note that even when the 3671κTg-B cells were responsible for almost 10% of the B cell compartment, like in the κTg^{int} chimera, the amount 3671κTg-Ig is approximately 300 times lower than mice in which virtually all B cells carry the 3671 κ-light chain (3671κTg-F1). This difference could be explained by the difference in the amount of total Ig present in the sera of the 3671κTg-F1 versus the κTg^{low} and κTg^{int}. To address this question, the total Ig was estimated by measuring the levels of IgM and IgG in sera of the mixed BMC mice as compared to that of the 3671κTg-F1 mice. As shown in figure 4B and 4C, the Ig levels in κTg chimeras cannot be responsible for the lower levels of 3671κTg-Ig found in these mice. Figure 4B shows the total Ig, IgM and IgG in the groups. The κTg chimeras have in average 3 times more total Ig than the 3671Tg-F1. There was no difference in the levels of total Ig between κTg^{low} and κTg^{int}. The ratio between 3671κTg-Ig and total Ig is shown in figure 4C. Since the total Ig levels were ~ 3 fold lower in the 3671κTg-F1 group than the κTg chimera, the percentage of 3671κTg-Ig in the sera of κTg chimeras were even lower

than predicted by figure 4A (1000 fold lower for the κTg^{low} and 300 times lower for the κTg^{int}).

3671κTg-B cells Show Normally Distribution in the B cell Areas

Since the ratio of 3671κTg-Ig total Ig was lower than predicted for the κTg chimera mice, it was speculated that maybe this was due to a possible defect in B cell localization in the follicules of an irradiated mouse. To address this question, we performed an immunofluoresce on frozen spleen sections of chimera mice. Figure 5 shows the results, where the control mice represent the background staining for the 1763 antibody (blue), and B220 defines the B cell follicule (red in the pictures). As predicted by the flow data, the κTg^{int} chimera mice have more 1763+ B cells than the κTg^{low} mice. It is important to note that the 1763+ B cells are randomly distributed throughout the B cell follicule in both κTg chimeras. Another important characteristic is the presence of 1763+ bright cells in the red pulp of the spleen, indicating that the 3671κTg-B cells are differentiating in 3671κTg-Ig secreting plasma cells. This indicates that there is no intrinsic defect with the 3671κTg-B cells in the κTg chimeras.

Arrested Development of CA30$^{Thy1.1}$ T cells in the Thymus of κTg Chimera Mice

The offspring of the CA30 and 3671κTg animals generate a double transgenic mouse in which the CA30 T cells are deleted in the double positive stage. To test how these T cells behave in animals where the 3671κTg is present under more physiological conditions, we examined the Thymus of the κTg chimera mice. Figure 6 shows a representative flow data from the Thymus of the chimera mice. The CD4 by CD8 graphs are shown in a Thy1.1+ gate. The control represents the CA30 $^{Thy1.1}$ T cells that developed in a host in which no 3671 peptides were present. As previously described this Tg has a tight allelic exclusion, where virtually no CD8 single positive (SP) T cells are encountered

and most of the transgenic T cells are in the CD4 SP stage. When the Thymus of either κTg^{low} or κTg^{int} was compared to the control chimera mice, fewer Thy1.1+ cells were detected (data not shown). When the Thy1.1+ compartment was compared to the κTg and the control chimera groups, most of the Tg T cells were arrested between the double negative and double positive stage. However, in contrast to the (CA30x3671κTg) F1, the Tg T cells were able to fully develop in CD4+ T cells. The number of transgenic T cells able to fully mature in the κTg chimera mice was inversely correlated with the 3671κTg-B cell frequency, where κTg^{low} contained more mature Thy1.1+ T cells than κTg^{int} chimera mice.

Mature Thy1.1+ T cells in the periphery of BMC mice

Since Ig specific T cells can recruit B cells in an autoimmune reaction that resembles SLE, we decided to look for the T cell phenotype in the periphery of the BMC mice. Figure 7A shows the percentage of Thy1.1+ CD4+ (CD16/32 – gate) cells in the experimental groups. As shown, very few mature Thy1.1+ T cell are encountered in periphery of the κTg chimera mice, though these numbers are significantly higher than the background stain (B6AF1). The Thy1.1+ T cells in the κTg chimera are a heterogeneous population represented by the CD4 stain, where the majority of the T cells are CD4low as compared to the control. Similar to the Thymus, the numbers of Thy1.1+ T cells are inversely correlated to the 3671κTg-B cell frequency, where κTg^{low} have more transgenic T cells than the κTg^{int} chimera group. Due to the fact that the presence of Thy1.1+ T cells in the periphery by itself does not indicate that the T cells are specific to the 3671 peptide, we stained spleen cells with the Tet IAk-3671. As shown in figure 7B, the T cells (Thy1.1+CD4+CD16/CD32- gate) in the κTg chimera groups were negative for the CA30 transgenic TCR (κTg^{low} represented by the solid line, κTg^{int} by the dotted line and control by the solid histogram). The Thy1.1 + T cells were also discriminated based on the CD4

profile, and no difference was seen in the Tet IAk-3671 stain between the CD4 high and low Thy1.1+ T cells (data not shown). Although none of the Thy1.1+ T cells were Tet IAk-3671+, that did not necessarily mean that those cells were not expressing the CA30 TCR Tg. The transgenic T cells may have down regulated the TCR after encountering the 3671 peptide in the periphery. An alternate hypothesis is that the transgenic T cells escaped negative selection in the Thymus and survived in the periphery through an expression of a secondary α-chain, since allelic exclusion is not tightly regulated for the α-chain(18). To distinguish between the two hypotheses, an anti-Vβ8 antibody (the β chain used by the CA30 TCR Tg) was used to detect the presence of the TCR on the surface of the T cells. As represented by figure 7C, the majority of the T cells were Vβ8 positive, at levels comparable to the control group. This data associated with the negative surface stain for the Tet IAk-3671, strongly suggests that the CA30 T cell escapees are a product of T cells that express a secondary α-chain. A fraction of the transgenic T cells in the κTg chimera (figure 7C) were Vβ8 negative, suggesting that some T cells completely down regulated the TCR. Another explanation is that the Thy1.1+ T cells are T cells that express a different β-chain. These two hypotheses are not mutually exclusive, and further research is needed to address this issue.

Thy1.1+ T cells Show a Phenotype of Antigen Experienced T cell but no Anergy Marker

To address whether the Thy1.1+ T cells encountered in the periphery were antigen experienced, we looked for the CD44 expression in these cells. As shown by figure 7D, the Thy1.1+ T cells in the κTg chimera were CD44 high as compared to the control group (solid histogram), indicating that Thy1.1+Tcells had encountered their cognate antigen. Because 3671-Ig peptides were present in our system during the development of the $CA30^{Thy1.1}$ T cells in a tolerogenic form, we looked for markers of anergy or a regulatory

phenotype. The CD5 antigen, a modulator of the TCR signal has been previously described in other models as being an important factor in the unresponsiveness of autoreactive T cells(19, 20). As shown by figure 7E, there was a small upregulation in CD5 expression between mixed BMC groups. We also looked for PD-1 expression, and as with the CD44 data, the κTg chimeras were high for PD-1 when compared to the control group seen (data not shown).

Thy1.1+ T cells Are Refractive to 3671-Ig Peptide Stimulation in vitro

To test if the transgenic Thy1.1+ T cells retain their ability to respond to 3671-Ig peptides, purified T cells from κTg and control chimeras were stimulated in vitro with 1μM of 3671-Ig peptide. Figure 8A shows proliferation of purified T cells corrected for the number of Thy1.1+ T cells in the culture. Both κTg chimeras (low or int), had no thymidine incorporation above background, suggesting that the Thy1.1+ T cells present in the periphery were unable to respond to a high peptide stimulation. The unresponsiveness of the T cells to the 3671-Ig derived peptide may be a result of the presence of regulatory T cells in the culture, in addition to a low frequency number of Thy1.1+ T cells. To explore this theory, purified T cells depleted of CD25+ were CFSE labeled and stimulated in vitro with 3671-Ig peptide. As shown in figure 8B, Thy1.1+ T cells from κTg^{low} or κTg^{int} chimera did not proliferate in vitro in response to peptide stimulation. This data suggests that even though the CA30 T cells reveal an antigen experienced phenotype, they are rendered tolerant *in vivo*.

Discussion

Previous literature attempting to explain the underlying mechanisms by which T cells are rendered tolerant to Ig specific peptides have failed to recapitulate the Ig diversity scenario. Our research developed a unique system to address the mechanisms underlying T cell tolerance to Ig derived peptides, where the frequency of a specific B cell resembles the natural B cell repertory. To do this we used a complementary pair of Tg mice previously characterized(16) as the CA30 TCR Tg mouse and 3671-κTg mouse. Using a mixed BMC approach, we showed that the majority of the Tg T cells were arrested in the Thymus of the animals carrying the 3671-κTg B cells. We also showed that a percentage of the Tg T cells fully mature in CD4+ T cells, and are able to reach the secondary lymphoid organs. The Tg escapees are antigen experienced and tetramer IAk-3671 negative, Vβ8+ and do not proliferate to 3671 peptide stimulation *in vitro.*

Sirisinha and Eisen using isologous mAb immunizations followed by the generation of T cell hybridomas demonstrated that T cells can be generated against Ig derived peptides (3), suggesting that T cell were not rendered tolerant to Ig sequences. Only after the advances in sequencing techniques and careful peptide mapping screens, was it determined that the T cells were not Ig germline specific. The T cells against the mAb were indeed responding to the somatic mutated regions, excepting only CDR3, where mutations and junction diversities are virtually indistinguishable (8-15).

The hybridoma approach is very helpful in the understanding of Ig tolerance, but lack in resolution to address the means by which T cell tolerance is achieved. Using the IgG2a$^{a/b}$ system, Bordenave and colleagues demonstrated that haplotype specific T cells are rendered tolerant by clonal deletion in the Thymus. The authors also showed that this deletion correlated with IgG mRNA expression in the Thymus, and with deletion of B cells from the mouse, T cells became responsive to Ig peptides(21). Supporting the idea that

tolerance is achieved in the Thymus, two independent models using TCR transgenic animals against Ig derived peptides demonstrated that the T cells were rendered tolerant by negative selection in the Thymus(16, 17). In contrast to these studies however, Granucci et al demonstrated that Tg T cells specific to the IgG2a^{b} haplotype escape tolerance in mice expressing endogenous levels of IgG2a^{b}(22). The results presented in this paper support in part the idea that the Ig specific T cells are rendered tolerant in the Thymus through clonal deletion. In our model, where the B cell frequency and serum Ig levels were more physiological, the majority of CA30 T cells were arrested between the double negative and double positive stage in the Thymus. However, unlike other models, the 3671-Ig specific T cells were not completely deleted, and some of the Tg T cells were able to mature to the CD4+ SP stage. The differences seen in the thymus from the mixed BMC and the work previously done by both our lab and by Bogen et al may be explained by the antigen levels present in each system. In previous experiments, the T cell epitope was present in massive amounts (milligrams per mililiter of sera) in the animal, and deletion is not surprising based on the early works showing thymic tolerance to ubiquitous expressed antigens(23). Using the mixed BMC approach we were able to generate animals in which the frequency of a specific B cell was less than 3% of the total B cells, compared to almost every B cell in previous work from our lab. The ability to generate that low frequency is critical for our studies, since a particular V gene might be present only once every 100 B cells (there are approximately 100 VH genes in mice). Interestingly enough, it was observed that the levels of secreted 3671-Ig in sera of a mixed BMC were lower than predicted by the percentage of 3671 circulating B cells in the κTg chimeras. This discrepancy can be explained by the lower representative frequency of 3671-κTg B cells in the B1 and Marginal Zone (MZ) compartments. The B1 cells are thought to develop mostly during the fetal liver stage, and have the ability to self-renew in the periphery(24, 25). The B1 and MZ are responsible for the early IgM B cell response to T dependent and

independent antigens(26). Because the κTg chimeras have more total Ig than unmanipulated 3671-κTg animals, it is very unlikely that the reconstituted chimera was deprived of the B1 lineage B cells. Histology in the spleens of κTg chimeras revealed normal B cell distribution in the B cell area, suggesting that the 3671-κTg B cells were distributed within the B cell follicle and MZ. The presence of bright spots in the red pulp of the spleen strongly suggests that some 3671-κTg B cells were recruited in an immune response and differentiated into plasma cells. This data suggests that there are no intrinsic defects with the 3671-κTg B cells in the κTg chimeras.

Analysis of the Tg T cells in the periphery revealed an intriguing phenotype. Although the Tg T cells found in the periphery were CA30 TCR Tg negatives, the vast majority were positive for the Vβ8 stain. Because our TCR Tg mouse was not in a RAG knock-out background, and the α-chain allelic exclusion is not as tightly regulated as the β-chain, the most likely explanation is that the Tg escapees were a byproduct of an allelic inclusion in which only the T cells expressing a secondary light chain escaped negative selection(18, 27-30). The idea of dual receptor lymphocytes and their potential harm to the organism has always been an interesting question. Zal et al used a diabetogenic TCR to show that autoreactive T cells were able to escape through the expression of a second α-chain. They also showed that once in the periphery, the dual receptor T cells were capable of inducing destruction of the pancreas if the T cells were activated by the non-autoreactive TCR(31). Transfer of CA30 T cells into a 3671-κTg animal induced severe hypergammaglobulinemia with IgG anti-chromatin antibodies in a presentation similar to a Lupus-like disease(16). Neither hypergammaglobulinemia nor anti-chromatin antibodies were seen in the κTg chimeras, suggesting that even though the Tg T cells were present in the periphery, they did not participate in a T dependent immune response with 3671-κTg B cells. Unlike the CA30 Tg T cell transfer into 3671-κTg B cells, adoptive transfer of both Tg cells into a non-transgenic host leads to a phenotype that is characterized by an early

antibody response from the 3671-κTg B cells followed by the disruption of the germinal center reaction and suppression of the 3671-κTg antibodies (unpublished data). This model may explain why the Tg T cells showed an antigen experienced phenotype (CD44 high) as well as the low amounts of 3671-Ig found in sera as a consequence of T-independent responses. Suppression of a specific Ig isotype was also seen in the IgG2a^{b} TCR Tg T cells system(22).

Chronically stimulated T cells or the presentation of antigens in absence of co-stimulation can lead T cells to a state of unresponsiveness, characterized by the up-regulation of CD5(19, 20, 32). In our model, the Tg T cells up-regulated the CD5 molecule or the other known marker of chronically stimulated T cells; the PD-1 surface protein(33, 34). In agreement with that Ig specific Tg T cells that develop in the presence of the cognate peptide were unable to proliferate in vitro. There are several plausible explanations for why the T cells were peptide refractive. One explanation is that Tg T cells silenced the Tg α-chain, and only expressed the endogenous α-chain. This is unlikely due to the fact that all the Tg T cells were antigen experienced. A second explanation is that the low levels of CD4 in the Tg T cells may affect the TCR signaling. The CD4 molecule increases the affinity of TCR for the peptide-MHC class II complex, and is also is responsible for bringing Lck close to the TCR-CD3 complex(38-40). A third hypothesis is that the Tg T cells were a dying population *in vivo* and that would explain why they did not proliferate. However, detection of Tg T cells after 5 days of stimulation suggests that the Tg T cells were a viable population

An interesting report by Naji et al demonstrated the presence of a CD3+CD4 low T cell population in the blood of transplanted patients(41). These cells were correlated with successful allograft transplantation, suggesting a role in suppression of immune responses through the production of IL-10. Further studies should be performed in our system to test if the Tg T cell escapees have a suppressive phenotype.

Though it is clear that Ig specific T cells are silenced in our system, the type of cell inducing tolerance is unknown. Some might speculate that soluble Ig is responsible for the effects seen in our system (indirect presentation), where DCs would have presented the 3671-Ig derived peptides in the Thymus and in the periphery, in a non-immunogenic way. This idea is supported by previous work showing that maternal IgG can induce tolerance(16). Following this idea, Hannestad and colleagues demonstrated that IgG mAbs are not immunogenic. Although it seems plausible that tolerance is achieved by soluble Ig, it is interesting to speculate whether it is the only source of tolerance. Studies done by Parker and colleagues suggested that resting B cells can induce tolerance to a soluble antigen through the generation of T reg(44). Additional evidence that B cells may be important in tolerance lies in the presence of the 3671-κTg B cell in the Thymus of mixed BMC mice, a phenomenon that also correlates with tolerance seen in the $IgG2a^b$ system. Arguing against this idea is the report by our lab that 3671-κTg resting B cells *in vitro* did not present peptides derived from the Ig(45). An interesting hypothesis is that anergic 3671-κTg B cells can induce tolerance in the T cells. The anergic B cells present a partially activated phenotype (CD80 high/ CD86 negative/ Fas positive), and can present 3671-Ig derived peptides. This hypothesis is being currently tested in our lab(45-47).

The importance of understanding the mechanisms by which T cells are rendered tolerant to Ig derived peptides may shed light on understanding of pathological immune responses. Several independent reports suggest that Ig specific T cells may play a role in the development of autoimmune diseases where autoantibodies are important.

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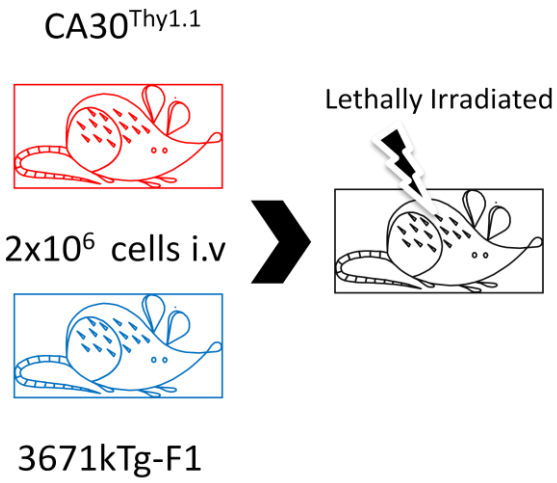
Figure 1



BMC	3671 κTg-F1	CA30$^{Thy1.1}$
κTg^{low}	6%	94%
κTg^{int}	12%	88%
control	---	100%

Figure 2

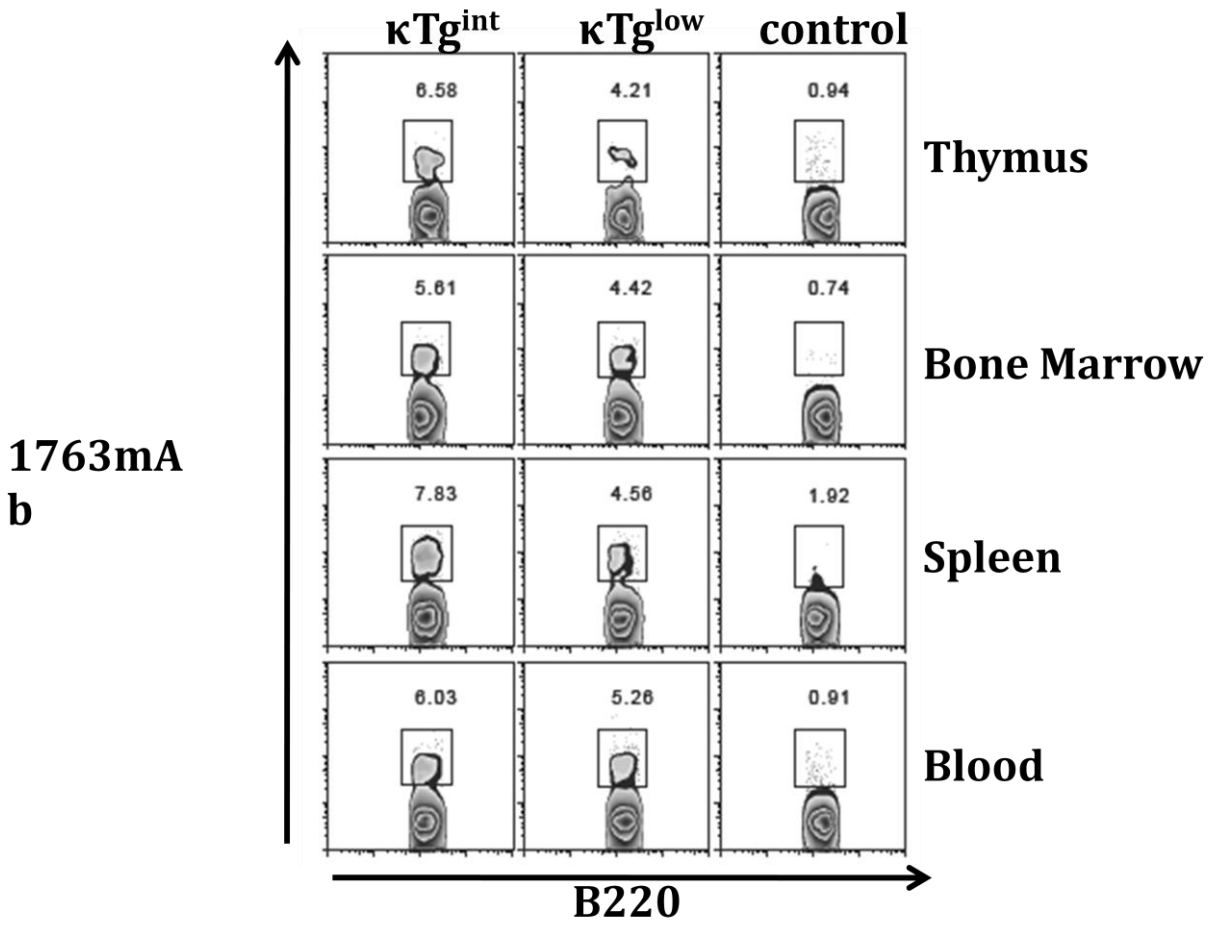

κTg^int
κTg^low
control
6.58
4.21
0.94
Thymus
5.61
4.42
0.74
Bone Marrow
7.83
4.56
1.92
Spleen
6.03
5.26
0.91
Blood
1763mA
b
B220

Figure 3

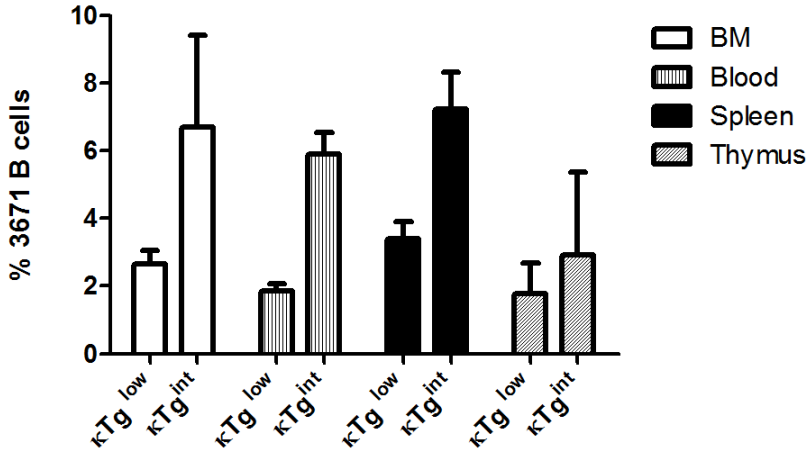

% 3671 B cells
10
8
6
4
2
0
κTg low
κTg int
κTg low
κTg int
κTg low
κTg int
κTg low
κTg int
BM
Blood
Spleen
Thymus

Figure 4

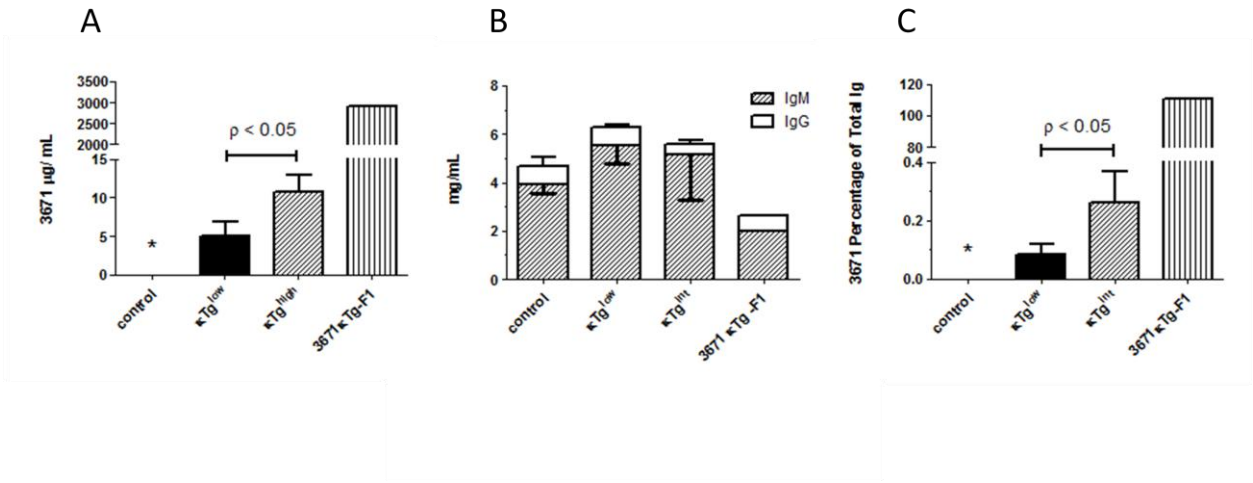

A
B
C
3671 µg/ mL
3500
3000
2500
2000
15
10
5
0
p < 0.05
*
control
κTg low
κTg high
3671κTg-F1
mg/mL
8
6
4
2
0
IgM
IgG
control
κTg low
κTg int
3671 κTg -F1
3671 Percentage of Total Ig
120
100
80
0.4
0.2
0.0
p < 0.05
*
control
κTg low
κTg int
3671κTg-F1

Figure 5

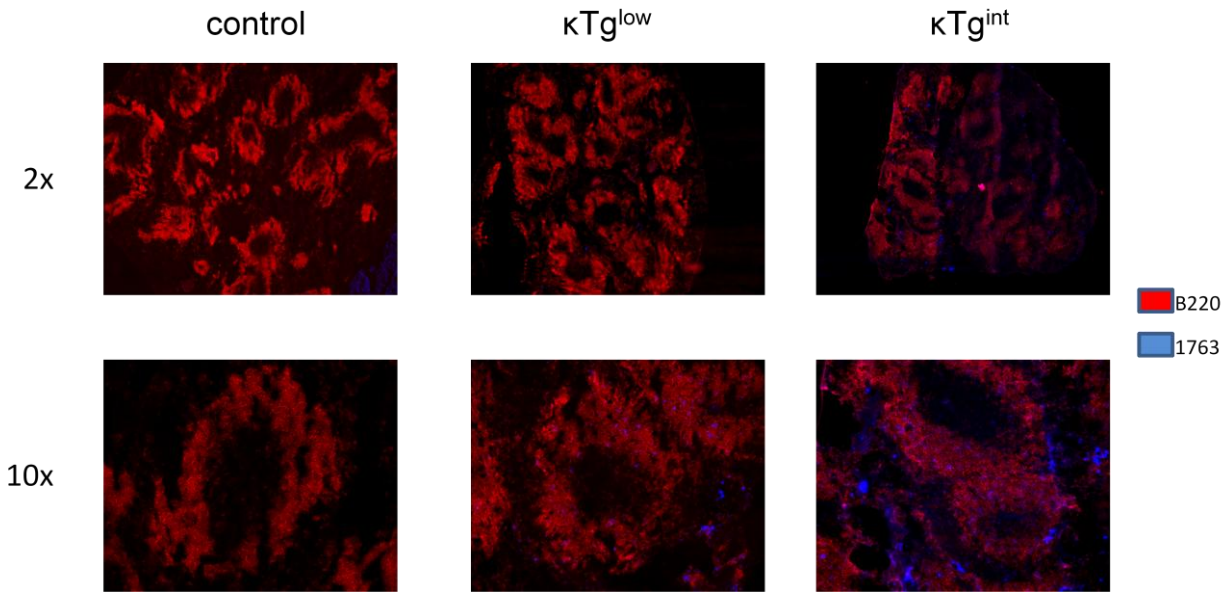


Figure 6

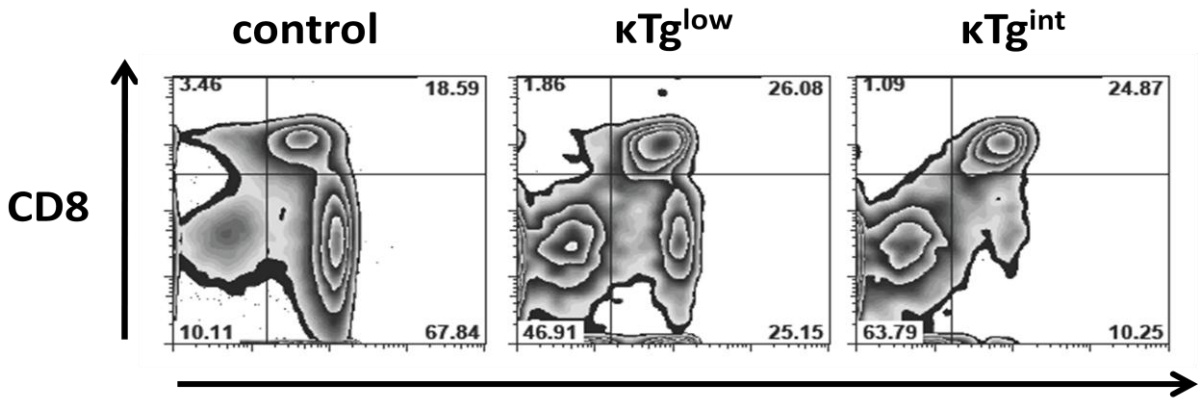
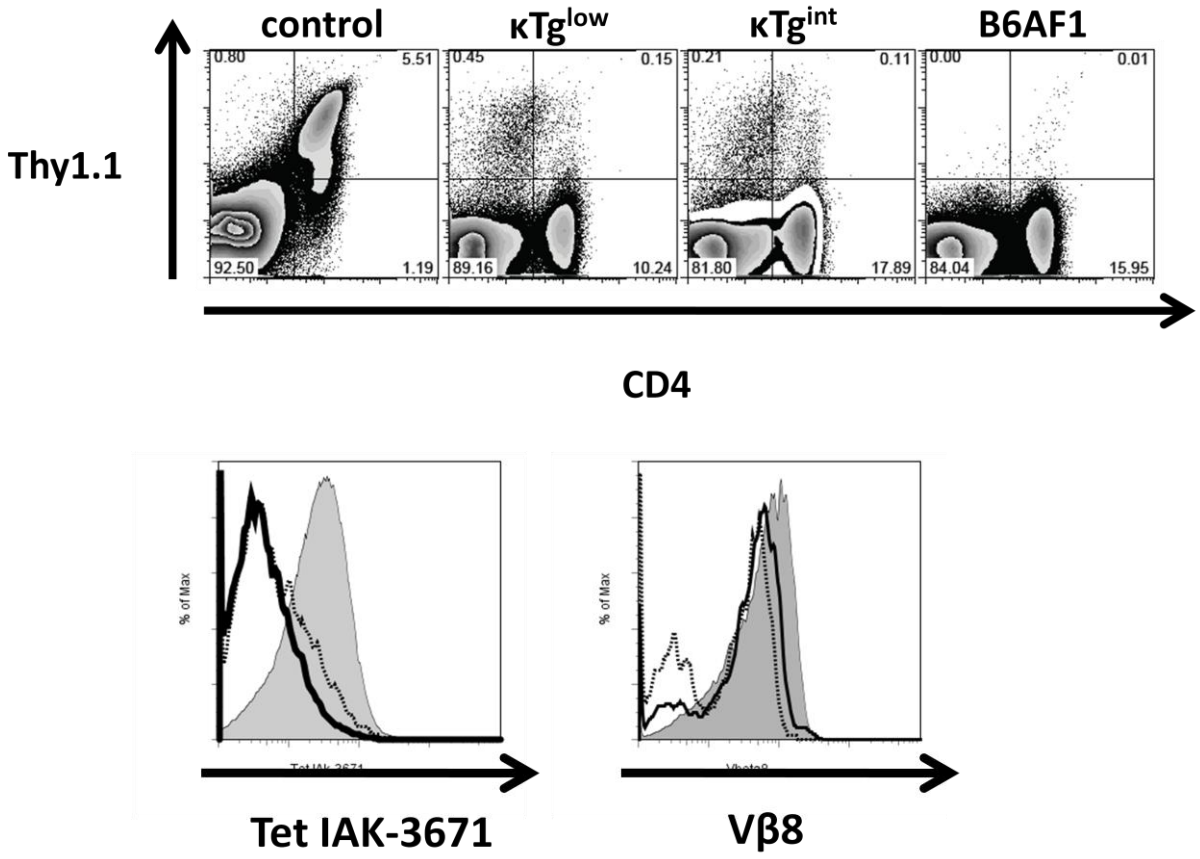

control
κTglow
κTgint
CD8
3.46
18.59
10.11
67.84
1.86
26.08
46.91
25.15
1.09
24.87
63.79
10.25

Figure 7


control
κTg^low
κTg^int
B6AF1
Thy1.1
0.80
5.51
92.50
1.19
0.45
0.15
89.16
10.24
0.21
0.11
81.80
17.89
0.00
0.01
84.04
15.95
CD4
% of Max
Tet IAK-3671
% of Max
Vβ8

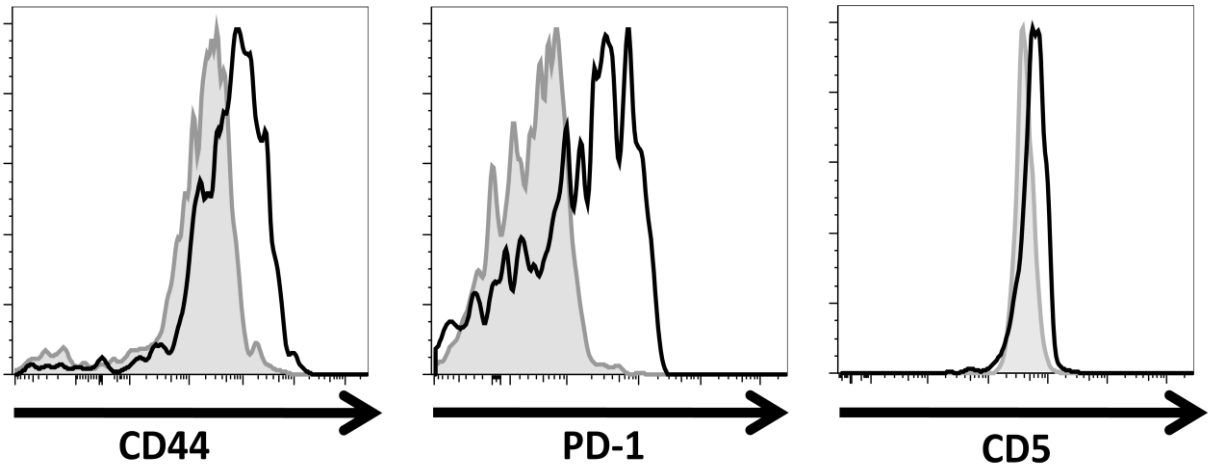

CD44
PD-1
CD5

Figure 8

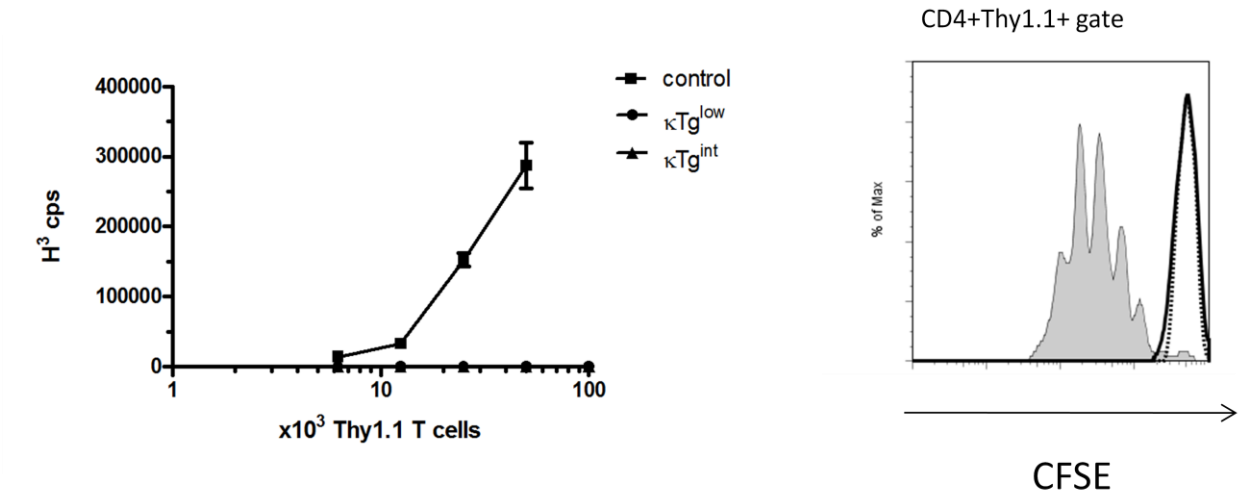

400000
300000
200000
100000
0
H3 cps
1
10
100
x10³ Thy1.1 T cells
control
κTglow
κTgint
CD4+Thy1.1+ gate
% of Max
CFSE

Table I

Lymphocytes	Marker	B6AF1^{recip}	3671 κTg-F1*	CA30$^{Thy1.1*}$
B cells	1763	-	+	-
T cells	Thy1.1	-	-	+
	IAk-3671	-	-	+

recip: stains for recipients mice

*: indicates donor mice

From: lupusjournal@compuserve.com

To: wysockil@njc.org

Cc:

Subject: Lupus - Manuscript ID LUPUS-08-014

Body: 09-Jan-2008

Dear Dr. Wysocki:

Your manuscript entitled "Spontaneous Autoimmunity in Mice That Carry an IghV Partial Transgene: A Required Arginine in VHCDR3" has been successfully submitted online and is being considered for processing.

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Denzil Fletcher

Lupus Editorial Office

Date Sent: 09-Jan-2008

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Cc:

Subject: Lupus - Decision on Manuscript ID LUPUS-08-014

Body: 18-Feb-2008

Dear Dr. Wysocki:

Manuscript ID LUPUS-08-014 entitled "Spontaneous Autoimmunity in Mice That Carry an IghV Partial Transgene: A Required Arginine in VHCDR3" which you submitted to Lupus, has been carefully considered by the Editorial Office along with outside reviewer(s) whose comments suggesting a MAJOR REVISION are included at the bottom of this letter.

I would be happy to reconsider your paper if you could modify your manuscript according to their comments and recommendations and make the major revisions suggested.

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With best regards.

Yours sincerely,

Graham Hughes

CAPÍTULO III

Spontaneous Autoimmunity in Mice That Carry an *IghV* Partial Transgene: A Required Arginine in VHCDR3

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Abbreviations: **SLE**: systemic lupus erythematosus. ***pTg***: partial transgene

Abstract

Systemic lupus erythematosus (SLE) is a prototypical systemic autoimmune disease, typically characterized by the presence of IgG autoantibodies directed against nuclear antigens. Here, we describe a spontaneous mouse model of autoimmunity resembling SLE, which occurs on a nonautoimmune-prone SWR genetic background. In this model, the SWR mice carry an *IghV* partial transgene encoding only the heavy chain variable domain of an antibody directed against chromatin. SLE-like disease in partial transgene mice was manifested by anti-nuclear antibodies, glomerulonephritis, splenomegaly and skin lesions, in various combinations among individuals. Disease was observed in three independent transgenic lines, but not in control lines carrying a partial transgene in which an Arg codon was converted to a Ser codon in VHCDR3 to ablate chromatin-reactivity. Disease was often but not always accompanied by anti-chromatin antibodies. Unexpectedly, the anti-chromatin antibodies detected in seropositive animals were not encoded by the partial transgene. These observations strongly implicate a role for the transgene product in disease initiation but not necessarily for end-state pathology, and they raise the possibility that in SLE, some autoreactive B cells may play an initiating role in the disease etiology.

Introduction

SLE is a systemic autoimmune disease or group of related diseases with numerous manifestations involving multiple organ systems that include the kidney, the skin and the lung (1). Much of the pathology is thought to be induced by immune complexes between autoantibodies and ubiquitous nuclear antigens. And many of the autoantibodies are derived from B cells that appear to be driven in a T cell-dependent immune response, indicating breaches in both T- and B-lymphocyte self-tolerance (2-5).

Although numerous self-antigens targeted by SLE autoantibodies have been defined, the antigenic specificity of T cell help for autoreactive B cells has proved to be elusive and controversial (6-10). Nevertheless, T cell help for autoreactive B cells seems to be a major rate-limiting step in disease. This can be inferred from studies in which anti-nuclear antibodies are induced when an arbitrary avenue of T cell help to anti-nuclear B cells is provided. Many investigators have reported this, even in mice that are not genetically predisposed to develop systemic autoimmunity (10-16). As such, it is tempting to conclude that the kinetics of autoantibody development in SLE is limited primarily or exclusively by the availability of autoreactive T helper cells.

On the other hand, it has proved more difficult to directly test whether the development of anti-nuclear B antibodies in SLE is also kinetically limited by the frequency of nuclear antigen-specific B cells in the repertoire. While it is clear that the B cell repertoire contains a substantial frequency of low-avidity self-reactive and often polyreactive members, it is unclear whether these are the antecedents of the T cell-dependent clones that eventually emerge in SLE and secrete anti-nuclear antibodies of high avidity (17-21). Studies in mice that carry Ig transgenes, encoding anti-nuclear antibodies have not unambiguously resolved this issue because the transgenic autoreactive B cells are subjected to self-tolerance mechanisms (21-23). This is probably why anti-nuclear antibody levels are relatively modest or even undetectable in mice that carry Ig trangenes derived from hybridomas producing anti-nuclear antibodies (24-29). In addition, it appears that many of the

autoreactive B cell clones that ultimately escape in such transgenic mice, edit their receptors by RAG-mediated recombination (24, 30-32). These observations suggest that immature bone marrow B cells that are “born” with an anti-nuclear B cell receptor (BCR) are not necessarily the immediate and unedited precursors of the high-avidity autoreactive clones that emerge in SLE.

The problem of not being able to increase the frequency of BCR-defined immunocompetent precursors to autoreactive clones is compounded by a lack of knowledge regarding exactly which subpopulation(s) of B cells are eligible, or at what stage of development they are eligible, for recruitment into an autoimmune response. A bone marrow B cell that is “born” with an autoreactive receptor must traverse all developmental stages and associated self-tolerance checkpoints to participate in autoimmunity. This could partly explain why mice that carry anti-nuclear Ig transgenes generally produce no or limited quantities of such autoantibodies. On the other hand, a B cell that acquires an autoreactive BCR via genetic means at a late stage in development would have to escape fewer tolerance checkpoints before differentiating into an antibody-secreting cell. Therefore, despite the relatively modest effects of Ig transgenes on autoantibody development, it is possible that a rate limiting step in autoimmunity is the presence of B cells with autoreactive receptors in a specific niche and at a particular moment. On the basis of this consideration, we asked what would happen if a B cell could recombine and express a VH/D/JH gene for an anti-nuclear antibody independently of the RAG-mediated mechanism that normally restricts this to pro- and pre- B cells.

To this end, we created mice carrying a *IghV* partial transgene encoding a VH/D/JH domain, derived from a hybridoma producing an antibody to a complex of histone 2A, 2B and dsDNA (H2A/H2B/dsDNA). Partial transgenes recombine into the *Igh* locus at a low frequency by homologous recombination in the JH intron to generate a complete functional Ig gene (33-36). Because the recombination mechanism does not require RAG enzymes, B cells that recombine and express a VH/D/JH partial transgene do not necessarily have to pass all of the developmental stages and tolerance checkpoints while expressing the transgene-encoded receptor.

We found that approximately one quarter of the partial transgene mice from 3 independent founders developed autoimmunity with manifestations of SLE-like disease. This disease occurred in mice of a nonautoimmune-prone SWR genetic background. It did not occur in 3 independent lines of SWR mice carrying a version of the partial transgene that was modified at one Arg codon previously shown to be essential for the chromatin specificity of the original monoclonal antibody. Unexpectedly, we could find no evidence that the transgene product was involved in end-state pathology, as might be expected of an autoantibody.

Materials and Methods

Mice

SWR/J were purchased from Jackson Laboratory. All mice were bred in our facility and used according to an IACUC approved animal protocol.

All *IghV* partial transgene (*pTg*) mice were initially generated on an SWR/J background, as described previously (33). Two versions of *pTg* mice were developed: *pTg18R* and *pTg104RS*. The *pTg18R* encodes the heavy chain V domain of an antibody specific for a complex of H2A/H2B/dsDNA. The original hybridoma (SN5-18) was generated from a spontaneously autoimmune (NZB x SWR)F1 mouse (3, 8). Two somatic mutations in the VH region that had no influence on chromatin-specificity were eliminated to produce *pTg18R* (8). In *pTg104RS*, an arginine codon at position 104 in CDR3 was additionally converted to a serine codon to eliminate chromatin specificity in the corresponding antibody (37). The partial transgenes contained approximately 1 kb of DNA upstream of the leader AUG start codon and approximately 1.6 kb of downstream DNA that included the JH cluster and the intron (mu) enhancer (Figure 1A). Three founder lines of SWR mice for each construct were obtained by injecting SWR eggs with *Eco*RI fragments of DNA (3 kb) devoid of bacterial sequences.

PCR and sequencing of genomic partial transgenes

Partial transgenes were amplified from genomic DNA in a nested PCR reaction, using primers Forward 1 (5'ACTGGCAAGGATTCACAGCAA 3') and Reverse 1 (5'GAAATGCAAATTACCCAGGTGG 3') for the first round, Forward 2 (5'ACTCTTCCATTGCTGGTGGGATTTC 3') and Reverse 2 (5'TTTGCTCAGCCTGGACTTTCGG 3') for the second round (Figure 1A arrows). All the reactions were performed using a high-fidelity DNA polymerase with proof-reading function, Phusion DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's specifications. The PCR products were extracted from an

agarose gel following electrophoresis and sequenced to confirm that they contained intact promoter and enhancer elements.

Assessing autoimmunity in partial Tg mice

Sera of partial transgene and wild type SWR mice were screened every three weeks for anti-chromatin antibodies and for physical signs of illness. The latter included skin lesions on the back of the neck and ears, splenomegaly (postmortem) and glomerulonephritis with Ig and complement factor 3 (C3) deposition (postmortem). Diseased animals, or animals that appeared healthy at 12 months of age were sacrificed, and organs were collected for further analysis. B cell hybridomas were generated from splenocytes of some animals using a standard procedure (38).

Kidney histology

Kidneys were frozen in Optimal Cutting Temperature (O.C.T.) Compound (Sakura Finekek Incorporated, Torrance, CA) or fixed in 2% formalin for at least 24 hours. Ig and C3 deposits were detected by immunofluorescence (IF), using FITC goat anti-mouse IgG or C3, using a in OCT frozen samples as described (10). Formalin fixed samples were stained with hematoxilin and eosin (H&E). All samples were blindly analyzed and scored.

Anti-nuclear antibodies

HEp-2 staining was performed on NOVA-Lite HEp-2 cells (Inova Diagnostics, San Diego, CA) using sera at a dilution of 1:50 followed by FITC goat anti-mouse IgG (heavy chain-specific) (Sigma, St. Louis, MO). Anti-chromatin IgG antibodies were detected by first coating 96-well microtiter trays with mouse chromatin (10 μg/ml), followed by incubation with blocking buffer solution [2 mg/ml bovine serum albumin (BSA), 1 mg/ml gelatin, 0.02% thimerosal, and 0.05% Tween-20 (Fisher Biotech, Fair Lawn, NJ)] at 37°C for 2 hours. Mouse sera diluted in blocking buffer (1:100) was added to the trays for one hour. IgG anti-chromatin antibodies were detected with a biotinylated goat anti-mouse IgG (heavy chain-specific) antibody (Southern Biotechnology

Associates, Inc., Birmingham, AL). Bound antibodies were quantified using a europium (Eu^{3+})-based fluorimetric immunossay as described (10), with one modification: the enhancement solution was prepared according to Keelan et al. (39). To control for nonspecific binding, serum samples were tested against BSA-coated trays and the bound radioactivity was subtracted from that of the chromatin-coated trays. Anti-chromatin antibody concentrations were calculated using the SN5-18 antibody to construct a standard curve (37). Total IgG antibodies were assayed in a similar manner, except that trays were coated with goat anti-mouse Ig (H+L) at 1 μg/ml (Southern Biotechnology Associates, Inc., Birmingham, AL). For chromatin-binding studies, of Figure 5C, IgG antibodies were purified and stripped of nuclear material as described (37).

A competition assay using an anti-clonotypic antibody (mAb7.4) was used to detect the presence of anti-chromatin antibodies encoded by *pTg18R*. MAb7.4 is specific for paired heavy and light chain variable domains of the SN5-18R mAb. 96-well trays, coated with mAb7.4 and treated with blocking buffer, were incubated with test sera (1:100) together with biotin-SN5-18R (0.5 μg/ml) followed by streptavidin-Eu^{3+} and enhancement solution. 50% competition was attained with 0.15-0.3 mg/ml of unlabeled SN5-18R.

IgG anti-chromatin subclasses

IgH isotypes of anti-chromatin antibodies were determined as above, except that antibodies were detected using horse radish peroxidase-conjugated goat antibodies against IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL) with the developing reagent, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). Samples were defined positive when the absorbance at 405 nm exceeded the average of negative controls plus 3 standard deviations.

Results

Spontaneous disease in SWR mice that carry an IghV partial transgene

We generated three independent lines of mice (*pTg18R*) carrying an *IghV* partial transgene encoding the heavy chain variable domain of an antibody directed against a complex of H2A/H2B/dsDNA. The original hybridoma producing this antibody was produced from a spontaneously autoimmune (NZBxSWR)F1 female mouse. As show in Figure 1A, the partial transgene construct contains approximately 1 kb of DNA upstream of the leader sequence and approximately 1.6 kb of DNA downstream of the assembled JH segment but lacks all constant region sequences. As such, only rare B cells with a partial transgene that has translocated into the *Igh* locus can express an antibody heavy chain with a variable domain specified by the partial transgene. Previous studies have shown that translocation occurs by homologous recombination at the 3' end of the partial transgene (35). However, such recombination was so rare that it could not be detected in *ex vivo* B cells. Instead, recombination was revealed in B cell hybridomas that were selected to express the partial transgene by an immunization strategy. For each *pTg18R* line, the partial transgene was amplified from genomic DNA (Figure 1B) and sequenced to confirm that promoter and enhancer elements were intact (data not shown).

When the first cohort of *pTg18R* mice aged beyond 5 months, some of the mice developed visible signs of chronic inflammation, as manifested by skin lesions and a scruffy appearance (Figure 1C). Control SWR mice in our colony never showed these signs of disease. Anti-nuclear antibodies were also present in sera of some animals, as seen in stains of HEp-2 cells (Figure 1D) and in chromatin-binding immunoassays. All mice with visible evidence of disease or chromatin-binding antibodies also had enlarged lymph nodes and spleens (Figure 1E), but not to the extent that they could be seen from the exterior. Table 1 lists various disease manifestations, distributed among affected individuals, together with the age of onset and gender. These first data suggested that there was no female sex bias among affected mice, an interpretation confirmed by subsequent cohorts of *pTg18R* mice.

SLE-like disease with kidney pathology

Because kidney pathology is a key manifestation of SLE, we examined kidneys of *pTg18R* mice that exhibited at least one other feature of disease (anti-chromatin IgG, skin lesions or splenomegaly). As predicted, most of these animals demonstrated signs of kidney disease, including deposits of IgG and/or C3, which were present in glomeruli of most mice with IgG anti-chromatin (Table 2). Lymphocyte infiltration was not as frequent, although it was observed in one diseased *pTg18R* mice that had no detectable levels of IgG anti-chromatin.

A CDR3 Arg is required for development of disease

Because disease developed in three independent lines of *pTg18R* mice and because it occurred in mice that were hemizygous with respect to the transgene, we infer that disease was not likely due to gene disruption at genomic sites of transgene integration. Given the origin of the partial transgene, we conjectured that development of disease was dependent upon the anti-nuclear specificity of antibody encoded by partial transgenes that recombined into the *Igh* locus. To test this idea we created three more lines of SWR mice with the same partial transgene in which a CDR3 Arg codon was converted to a serine codon. This alteration ablates the chromatin specificity of the original monoclonal antibody. As before, we amplified and sequenced the partial transgenes (*pTg104RS*) from genomic DNA to confirm that promoter and enhancer elements were intact.

Several cohorts of *pTg104RS* mice and *pTg18R* and SWR mice were allowed to age until they developed disease, as assessed by visible appearance or anti-chromatin antibodies, or until 12 months if no visible signs of disease were present. Animals were then sacrificed and their spleens were assessed for mass. While 23% of *pTg18R* mice showed clear signs of disease, none of the *pTg104RS* mice or SWR mice showed any sign at one year of age. There was no significant gender bias among afflicted *pTg18R* mice. Table 3 summarizes these results. The most common disease feature was splenomegaly followed by serum IgG anti-chromatin antibodies. On average, spleens of diseased *pTg18R* animals were twice the mass of those of *pTg104RS* or SWR mice or

of non-diseased *pTg18R* mice (Figure 3). No changes in the percentages of T and B cells subpopulations were seen in the spleens of diseased mice (data not shown). Kidneys of several aged *pTg104RS* were also analyzed for the presence of IgG immunedeposition, and none showed any difference when compared to the SWR control mice (data not shown).

Chromatin-reactive autoantibodies

We tested the anti-chromatin IgG for heavy chain isotype and found that the predominant subclasses were γ2a and γ3, followed by γ2b (Figure 4). This subclass distribution, which is indicative of inflammation, is similar to that observed in spontaneous models of SLE (1, 40). Sera of *pTg18R* mice that contained IgG anti-chromatin were further analyzed quantitatively in a fluoroimmunometric assay, as described in the Materials and Methods. The concentration of IgG anti-chromatin in these mice averaged 40 μg/ml, which was approximately 2-fold less than that in sera pooled from 10 autoimmune B6.*Sle1* mice. (Figure 5A). On average, *pTg18R* mice with IgG anti-chromatin also had total IgG concentrations that were 2-fold higher than non-diseased and control animals (Figure 5B).

To ensure that we were not misled by increased nonspecific binding to the assay trays due to increased concentrations of total IgG in diseased mice, we performed an additional test with serum IgG that was purified on a goat anti-mouse IgG affinity column. The purification procedure was designed to remove nuclear antigens from anti-nuclear antibodies because such immune complexes can produce artifacts in binding assays (37, 41, 42). Purified IgG was then tested at defined concentrations in the chromatin-binding assay. As shown in Figure 5C, the sera that tested positive for anti-chromatin IgG in the initial assay were positive again in the normalized assay using purified IgG. This result is consistent with idea that the anti-chromatin antibodies were products of specific clonal expansion, as opposed to polyclonal expansion/activation.

To determine if the anti-chromatin antibodies were encoded by the partial transgene, we tested sera of *pTg18R* mice in competition immunoassay using a monoclonal anti-idiotypic antibody

directed against the original SN5-18 monoclonal antibody. However, no such antibodies were detected in any *pTg18R* mouse sera. In addition, we generated 4 hybridomas producing anti-chromatin antibodies from 2 diseased *pTg18R* mice and sequenced their heavy chain variable regions. The sequences revealed that none of the hybridomas expressed the VH gene encoded by *pTg18R* (data not shown). Thus, we found that most diseased *pTg18R* mice produced anti-chromatin antibodies, but none of the mice had detectable levels of antibody derived from the partial transgene.

Discussion

We describe an SLE-like disease that develops spontaneously in SWR mice that carry a partial transgene encoding the heavy chain V domain of an anti-nuclear antibody with specificity for a complex of H2A/H2B/dsDNA. The disease is variously manifested among individuals by one or more of the following features: serum anti-chromatin antibodies, glomerulonephritis, skin lesions and enlarged secondary lymphoid organs, which collectively occur at a penetrance of ~20-25%. A majority of the anti-nuclear antibodies were of heavy chain isotypes (γ2a, γ3) indicative of inflammation and class switch recombination driven by IFN- γ. This, together with the other disease manifestations, such as skin lesions and enlarged lymphoid organs, indicate an inflammatory condition.

None of the autoimmune features observed in *pTg18R* mice were observed in similarly aged control wildtype SWR mice, which are not genetically-predisposed to develop autoimmunity. Moreover, it is unlikely that disease in *pTg18R* mice is due a genetic disruption at the site of transgene integration because disease occurred in hemizygous *pTg18R* mice, in all 3 lines of *pTg18R* mice, and not in any of 3 control *pTg104RS* lines. These observations indicate that *pTg18R* induces autoimmunity in mice that are not genetically predisposed.

We infer that protein product of the partial transgene initiates disease because a VHCDR3 arginine codon in *pTg18R* was required for pathology. This same Arg was also required for the chromatin-specificity of the original anti-nuclear monoclonal antibody that defines our system (37). Moreover, the SWR genome carries the same *Vκ10.2* gene that encodes the light chain V region of the original anti-chromatin monoclonal antibody (43).

It is unlikely that the untransloctated *pTg18R* directs synthesis of a protein product because mRNA lacking proper termination and polyadenylation sequences is highly unstable. We also considered the possibility that *pTg18R* integrated within the genome in such a manner as to encode a pathogenic fusion protein, but dismissed this for two reasons. First, such an event would have to

occur 3 times independently. Second, the recombination event(s) would have to be restricted to a 70 base segment of DNA between the disease-determining Arg codon at position 104 in CDR3 and an in-frame translation termination codon located just 12 bases downstream of the last JH codon. We considered the possibility that the NZB-derived *pTg18R* encoded an immunogenic VHCDR3 peptide (containing Arg_{104}) with respect to SWR CD4+ T cells and hence provided an avenue of help to B cells without recombining into the *Igh* locus. This is highly unlikely because of the mRNA instability issue mentioned above. Moreover, in a preceding study, immunodominant VH peptides of the 18R antibody (restricted by the SWR I-A^q molecule) were found in FR1 and CDR2 (43). Because these peptides are also encoded by *pTg104RS*, we would have expected autoimmunity in *pTg104RS* mice if T cell help to VH peptides were rate-limiting in disease initiation.

Our *IghV* partial Tg mice are unique among current Ig transgene models for autoimmunity because genetic recombination is required for expression of the heavy chain variable domain encoded by the transgene. Importantly, partial transgenes have no heptamer/nonamer recombination signal sequences, and studies in the anti-arsonate model have confirmed that partial transgenes recombine into the *Igh* locus by homologous recombination. (33-36). In principle, such recombination could occur at any stage of B cell development, even in a cell that already expresses an antigen receptor. The result of such a scenario would be similar to one in which a mature B cell in a nontransgenic mouse edits or revises its receptor without a deletion of the originally expressed allele (44-48). Evidence for receptor revision in peripheral B cells of autoimmune-prone nontransgenic mice was recently reported by Wakui et al. (49) and in autoimmune-prone Ig transgenic mice by two laboratories (50, 51).

Late-stage recombination of *pTg18R* into the *Igh* locus of peripheral B cells could explain why *pTg18R* mice develop autoimmunity despite the infrequency of such recombination events. Presumably these B cells would not have to traverse all stages of development and face the associated self-tolerance checkpoints that begin in the bone marrow. Late-stage recombination could also explain why there is no gender bias for disease in *pTg18R* mice. Estrogen relaxes negative selection of autoreactive B cells and is partly responsible for the female sex bias seen in

spontaneous human SLE or animal models of SLE (52, 53). However, estrogen may not be so critical if the B cell is already activated at the time when partial transgene rearrangement occurs. Regardless of why partial transgene mice develop autoimmunity, the induction of disease by a partial Ig transgene in mice that are not otherwise predisposed to autoimmunity is unique among current models of SLE.

Our findings suggest that immunocompetent precursors to anti-nuclear B cells can be rate-limiting with respect to the development of anti-nuclear antibodies in systemic autoimmunity. Although this idea is not widely accepted by investigators of SLE, there is some support for it in the literature. For example, Wang et al. (54) reported that a DNA mimetope could not induced anti-nuclear antibodies in DBA/2 mice, although it could in BALB/c mice. Similarly, we have found that when SWR mice are injected with calf chromatin, they develop strong IgG anti-chromatin antibodies that bind calf chromatin but not mouse chromatin (unpublished data). Both of these observations indicate that even though T cell help was available, potential anti-nuclear B cells could not be successfully recruited into an autoimmune response.

Partial transgenes translocate to the *Igh* locus at a very low frequency, such that very few B cells in *pTg18R* mice are expected to produce a receptor with the appropriate light chain that confers specificity for H2A/H2B/dsDNA. In fact, like Guisti et al. (35), we have not been able to detect the recombined partial transgene in central or peripheral lymphoid tissue by PCR of *ex-vivo* lymphocytes. Moreover, in another study involving a partial transgene encoding the VH of a rheumatoid factor crossed onto an MRL/lpr genetic background, the *pTg*-derived antibody could not be detected even though the repertoire of spontaneous autoantibodies was clearly altered (55). The low frequency of recombination could, in part, explain the low penetrance and delayed kinetics of disease in *pTg18R* mice. In addition, other regulatory mechanisms may functionally inactivate or physically remove most of the anti-nuclear B cells expressing the partial transgene. Hahn and colleagues reported evidence for regulatory CD8 T cells that are able to suppress autoreactive B cells, apparently in a manner that is specific for the B cell receptor (56). Cells of this type in the SWR strain conceivably could inhibit anti-nuclear B cells expressing the *pTg18R*, resulting in

marginal propagation of the B cells and a low penetrance of disease. Although some of our diseased *pTg18R* mice produced no detectable serum anti-nuclear antibodies, there are reports of seronegative SLE in which no anti-nuclear antibodies can be detected. This has been reported both in humans and in an MRL*/pr/lpr* mouse model in which B cells express a transgene-encoded Ig that cannot be secreted (57-59).

Collectively, our results support the idea that low frequencies of anti-nuclear B cells can initiate SLE-like disease in animals that are not genetically predisposed. They show that the product of the Ig transgene that initiates disease is not a major contributor to the anti-nuclear antibodies observed at late stages of disease. And they show that in some cases, the transgene product induces a seronegative form of SLE-like disease. Whether anti-nuclear antibodies or B cells cryptically initiate spontaneous SLE is unknown, but the possibility is raised by the seronegative feature of disease that is shared between our *pTg18R* mice and certain patients with SLE .

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Figure Legends

FIGURE 1. Partial transgene construct design and disease manifestations. *A,* Schematic illustration of *pTg18R* construct that was injected into fertilized SWR eggs and PCR products (*B*) resulting from amplification of genomic DNA with indicated primers (arrows in *A*), as described in Materials and Methods. Promoter (P), leader (L) and enhancer (E) positions are indicated (Figure is not to scale). Note that the constructs lack constant region exons. Example of skin lesion (*C*), nuclear staining of HEp-2 cells using sera (diluted 1/50) from one *pTg18R* animal (*D,* left panel) but not another (*D,* right panel) and splenomegaly (*E*).

FIGURE 2. Kidney immunopathology in autoimmune *pTg18R* mice. IgG and complement factor 3 deposition revealed by immunofluorescence, and glomerular inflammation revealed by H&E staining for representative diseased (left panels) and non-diseased (right panels) *pTg18R* mice.

FIGURE 3. Splenomegaly in autoimmune *pTg18R* mice**.** Average spleen masses are indicated for diseased *pTg18R* mice, and non-diseased *pTg18R, pTg104RS* and SWR mice. The spleens of *pTg18R* diseased animals were significantly more massive than those the control groups (* *pTg18R* diseased vs *pTg18R* nondiseased p= 0.0167, ** *pTg18R* diseased vs *pTg104RS* p= 0.003, *** *pTg18R* diseased vs SWR p= 0.009).

FIGURE 4. Heavy chain isotypes of IgG anti-chromatin antibodies in *pTg18R* mice. IgG Anti-chromatin antibodies in *pTg18R* sera were quantified with isotype-specific antisera. 1 unit (dotted line) represents the average for a pool of negative sera from SWR mice plus 3 standard deviations.

FIGURE 5. IgG anti-chromatin as a product of clonal selection. IgG anti-chromatin (A) and total IgG (B) in *pTg18R* sera were quantified as described in the Materials and Methods. Asterisk indicates that counts bound to chromatin-coated trays were less than or equal to zero after subtracting counts bound to BSA-coated control trays. B6.*Sle1* mice were 5 months old. *C*, IgG

anti-chromatin normalized to total IgG. Assay was performed with purified serum IgG that was treated to remove contaminating nuclear antigens. Results are expressed as micrograms of IgG anti-chromatin per milligram of total purified IgG to control for nonspecific binding as a function of total IgG.

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Figure 1

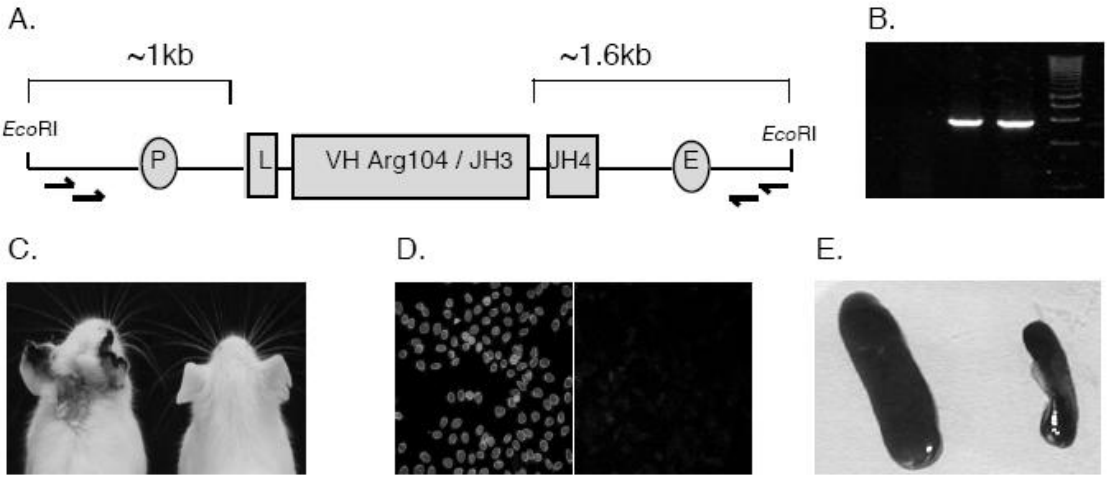

Figure 2

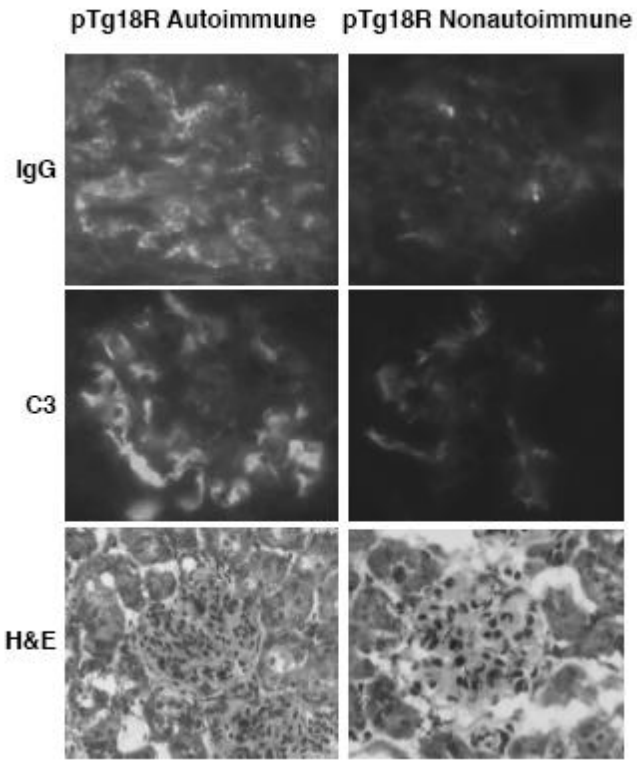

Figure 3

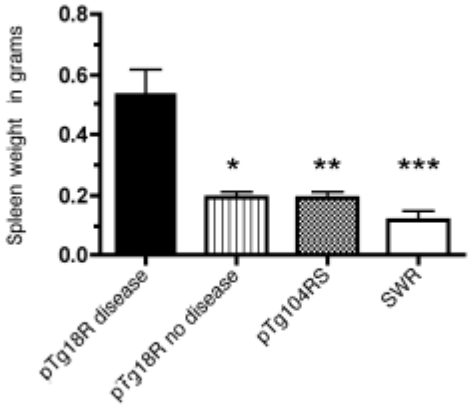
Spleen weight in grams
0.8
0.6
0.4
0.2
0.0
*
**
***
pTg18R disease
pTg18R no disease
pTg104RS
SWR

Figure 4

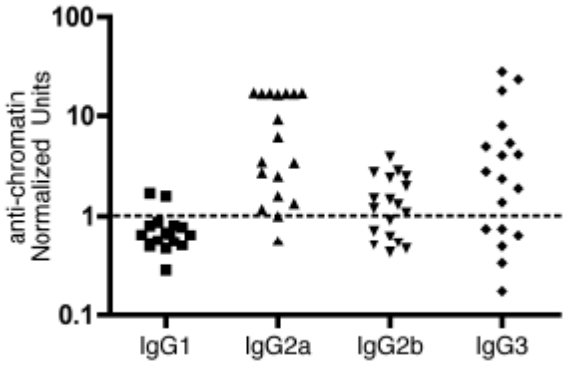
100
10
1
0.1
anti-chromatin
Normalized Units
IgG1
IgG2a
IgG2b
IgG3

Figure 5

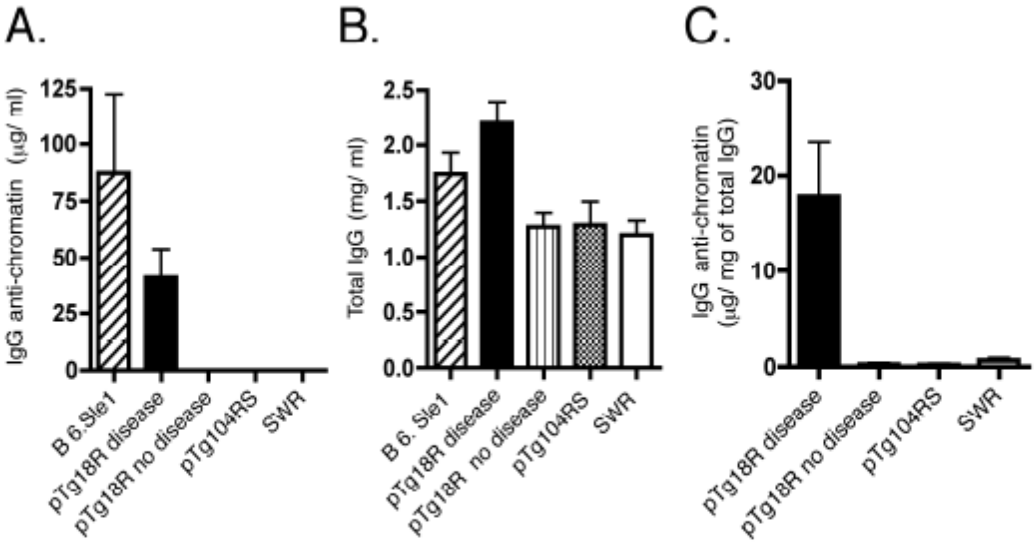
A.
IgG anti-chromatin (µg/ ml)
125
100
75
50
25
0
B 6.Sle1
pTg18R disease
pTg18R no disease
pTg104RS
SWR
B.
Total IgG (mg/ ml)
2.5
2.0
1.5
1.0
0.5
0.0
B 6. Sle1
pTg18R disease
pTg18R no disease
pTg104RS
SWR
C.
IgG anti-chromatin
(µg/ mg of total IgG)
30
20
10
0
pTg18R disease
pTg18R no disease
pTg104RS
SWR

Table 1. Manifestations of SLE-like disease in 3 lines of *pTg18R* mice

Line[a]	Sex	Splenomegaly	Skin lesion	IgG α-chr[b]	Age[c]
pTg18R.1	M	+	-	-	4.5
pTg18R.1	M	+	-	-	9
pTg18R.2	M	-	+	+	7
pTg18R.2	F	+	-	+	7
pTg18R.3	M	+	-	-	7.5
pTg18R.3	F	+	+	+	5

[a] Numbers after decimal point indicate independent lines of transgenic mice

[b] IgG anti-chromatin detected in sera

[c] Age in months at which first manifestation of disease was observed

Table 2. Kidney disease in *pTg18R* mice

pTg18R diseased mice[a]	IgG a-chr[b]	IgG[c]	C3[c]	H&E stain[d]
1	+	1-2,M	0	0
2	+	0	0	2, p, m
3	+	3, M	3, M	2, p, m, LI
4	+	0	0	0
5	-	2, P	3, P	2, p, m, LI
6	-	0	0	0
7	+	1-2, P	2, P	3, p, m
8	-	0	2, M	0
9	+	2, P, gbm	1, P, gbm	3, p, m
10	+	1-2, M	1, M	0
SWR				
1	-	0	0	0
2	-	0	0	0
3	-	0	0	0
4	-	0	0	0
5	-	0	0	0
6	-	0	0	0
7	-	0	0	0
8	-	0	0	nd
9	-	0	0	nd
10	-	0	0	nd

[a] These mice exhibited at least one of the 3 following signs of disease: IgG anti-chromatin, skin lesions or splenomegaly.

[b] IgG anti-chromatin detected in sera.

[c] Kidney sections were analyzed by immunofluorescence for IgG and C3 deposition. "M" indicates mesangial staining, "P" indicates peripheral capillary loop staining, "gbm" indicates staining clearly associated with glomerular basement membrane .

[d] Kidney sections analyzed in a H&E stain. "p" indicates peripheral involvement, "m" indicates mesangial involvement and "LI" indicates inflammatory cellular infiltrates including macrophages, lymphocytes and neutrophils.. "nd" indicates not analyzed by H&E stain.

Numbers indicate pathologic index where 0 is no disease and 4 is most severe.

Table 3. Frequency of SLE-like disease in pTg18R mice

	Disease	Spleen[b]	Lesion	IgG α-chr[c]
pTg18R combined	26/123 (21.13%)	24	9	18
pTg18R.1[a]	7/41	7	0	2
pTg18R.2[a]	5/18	3	3	4
pTg18R.3[a]	14/64	12	6	12
pTg104RS combined	0/114 (0%)	0	0	0
pTg104RS.1[a]	0/56	0	0	0
pTg104RS.2[a]	0/41	0	0	0
pTg104RS.3[a]	0/17	0	0	0
SWR	0/60 (0%)	0	0	0

[a] Numbers following decimal point indicate lines of *pTg18R* or *pTg104RS* mouse.

[b] Splenomegaly

[c] IgG anti-chromatin detected in sera.

CAPÍTULO IV

4. Considerações Finais

A habilidade das células T em reconhecer antígenos derivados da Ig e, através desse auxílio antígeno-independente em relação às células B, recrutar células B em respostas auto-imunes, sugere o quão perigoso é este tipo de interação entre os linfócitos T-B.

Nesta Tese, em uma tentativa de entender os mecanismos que governam e regulam este tipo de interação, foram criados dois modelos experimentais. O primeiro, com a finalidade de estudar os mecanismos que regulam a tolerância das células T a peptídeos derivados da Ig. O segundo modelo foi desenvolvido para estudar a apresentação do receptor como via para o auxilio de células T no SLE.

A primeira parte objetivou testar se as células T Ig específicas são tornadas tolerantes aos peptídeos derivados da Ig. Neste sistema, diferente dos demais usados na literatura, a expressão de um V gene estudado é mantido em freqüências próximas ao fisiológico. Como na maioria dos outros modelos, foi verificado que as células T Ig específicas são rendidas tolerantes aos peptídeos derivados da Ig. Entretanto, diferente dos modelos anteriores em que altas concentrações de anticorpo no soro estavam presentes, uma pequena porcentagem das células T Ig específicas escaparam à seleção negativa no timo. As células T encontradas na periferia apresentavam um fenótipo anérgico, representado pela incapacidade de proliferar *in vitro.* Estas células T apresentam marcadores de ativação e aparentemente escaparam à seleção negativa pela expressão de uma cadeia α secundária.

O segundo modelo desenvolvido teve como objetivo testar a hipótese da apresentação do receptor como via para o auxílio das células B auto-reativas no SLE. Neste modelo, se a apresentação do receptor fosse a viapela qual as células B são recrutadas em uma resposta auto-imune, mutações no receptor na região FR1 seriam encontradas; caso contrário, outras mutações seriam encontradas aleatoriamente. Entretanto, um resultado surpreendente aconteceu: aproximadamente 20% dos animais pTg18R, em uma linhagem não auto-imune, desenvolveram espontaneamente anticorpos anti-chromatina e uma doença semelhante ao SLE humano. Neste modelo, nenhuma das células B auto-reativas encontradas expressaram o Tg, indicando que a cadeia pesada 18R era importante para o início da doença, mas não necessária para sua manutenção. Devido à ausência de células B expressando a cadeia pesada 18R, foi impossível testar a hipótese da apresentação do receptor como origem do auxílio das células T. Apesar de este modelo não ter se mostrado útil para testar os objetivos inicias, usando o pTg18R, mostrou-se único em vários aspectos. Primeiro, este é o único modelo presente na literatura em que o SLE é induzido espontaneamente em uma linhagem não auto-imune de camundongos. Segundo, aparentemente, a presença de anticorpos em níveis baixos ou não detectáveis é capaz de induzir uma doença auto-imune. Terceiro, devido às únicas habilidades do pTg, células B podem adquirir um receptor auto-reativo na periferia, criando um modelo único para o estudo da Revisão do Receptor na periferia para a criação de uma célula B auto-reativa.

O melhor entendimento dos processos que controlam a resposta imune e os mecanismos pelos quais o sistema imune torna-se tolerante a antígenos próprios pode levar a melhorias no tratamento de diversas patologias. A indução ou quebra de tolerância a antígenos próprios são dois lados de um mesmo processo. O domínio deste mecanismo teria rápida influência na cura de doenças auto-imunes, como SLE, diabetes, esclerose múltipla, artrite, e transplante de órgãos, em que a indução à tolerância se faz necessária,

e também em doenças como câncer, em que a sua quebra poderia levar à erradicação do tumor.

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