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Mechanisms of Tolerance Induction to Foreign Proteins

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To both my grandmothers,

Manuela and Palmira

"...Sádico, às vezes é trágico, Mágico..."

Expensive Soul,

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Abbreviation List

4-1BB	CD137
Ag	Antigen
AHR	Airway Hyper Reactivity
AICD	Activation Induced Cell Death
Alum	Aluminum hidroxide
AOD	Autoimmune Ovary Disease
APC	Antigen presenting cell
B6.IL-10	IL-10 deficient C57Bl/6 mice
BAL	Broncho Alveolar Lavage
Bcl	B-cell Lymphoma
BM	Bone Marrow
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CIA	Collagen Induced Arthritis
CNS	Central Nervous System
CpG	Cytosine Guanine Dinucleotide
CTLA-4	Cytotoxic T-cell Associated Antigen
cTEC	Cortical Thymic Epithelial Cell
CXCR	Chemokine Receptors
DC	Dendritic Cell
DN	Double Negative CD4 CD8 cell
DP	Double Positive CD4 CD8 cell
DO11.10	OVA specific Balb/c mice
DST	Donor- Specific Transfusion
EAE	Experimental Autoimmune Encephalomyelitis
EAM	Experimental Autoimmune Myelitis
EAU	Experimental Autoimmune Uveoretinitis
ELISA	Enzime Linked Immuno Assay

Fab	Antigen-Binding Fragment
FcξR	FcR (Fragment, crystallizable) Receptor
Foxp3	Forkhead box P3
FVIII	Coagulation Factor VIII
GATA3	Trans-acting T-cell-specific Transcription factor
GC	Germinal Center
GITR	Glucocorticoid Induced TNF Related protein
HA	Hemaglutinin
HDM	House Dust Mite
HemA	Hemophilic
hFIX	Recombinant Human Factor VIX
hFVIII	Recombinant Human Factor VIII
i.m.	Intra muscular
i.n.	Intra nasal
i.p.	Intra peritoneal
i.v.	Intra venous
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecules
ICOS	Inducible T-cell Costimulator
IFA	Incomplete Freund's Adjuvant
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cells
iNKT	Induced NKT
ITI	Immune Tolerance Induction
iTreg	Induced Treg
K/BxN	G6PI transgenic TCR mice
LCVM	Lymphocytic Choriomeningitis Virus
LFA	Lymphocyte Function-Associated Antigen
LFOT	Low Frequency Forced-Oscillation Technique
LN	Lymph Node
MAb	Monoclonal Antibody
MBP	Myelin Basic Protein

MCh	Metacholine
MHC	Major Histocompatibility Complex
MLN	Mediastinal Lymph Nodes
MRL/lpr	Transgenic mice Defective in Fas mediated apoptosis
MS	Multiple Sclerosis
mTEC	Medullary Thymic Epithelial Cell
NFAT	Nuclear Factor Of Activated T-Cells
NKT	Natural Killer T-cell
NOD	Non Obese Diabetic
nTreg	Natural Treg
OIT	Oral Immuno Therapy
OT II	OVA specific C57Bl/6 mice
OVA	Ovalbumin
OX40	CD134
PBS	Phosphate Buffer Solution
PLP	Proteolipid Protein
PR8	Murine Influenza
RA	Rheumatoid Arthritis
Rag	Recombination Activating Gene
Raw	Airway Resistance Ag – Antigen
RORy	RAR-related Orphan Receptor Gamma
Runx1	Runt-related Transcription Factor 1
S.c.	Sub cutaneous
SCIT	Subcutaneous Immunotherapy
SDS	Sodium Dodecyl Sulfate
SIT	Allergen Specific Immunotherapy
SKG	Mice with a mutation in the Zap-70
SLE	Systemic lupus erythematosus
SLIT	Sub-lingual Immunotherapy
SP	Spleen
SPF	Specific Pathogen Free
STAT	Signal transducer and activator of transcription
T/R	Rag deficient MBP specific TCR transgenic mice

TACI	Tumor necrosis factor receptor superfamily member 13B, also known as TNFRSF13B
T-bet	T-box Transcription Factor
T-Bmc	Transgenic mice with a T cell receptor specific for OVA and a B cell receptor specific for HA
TCR	T-Cell Receptor
Tfh	Follicular T helper Cell
TFR	Follicular T regulatory Cell
TGF-β	Transforming Growth Factor Beta
Th	T Helper Cell
TIM	T-Cell and mucin domain containing molecule
TNF	Tumor Necrosis Factor
Treg	Regulatory T Cell
TSLP	Thymic Stromal Lymphopoietin
Wk	Week
ZAP-70	Zeta-chain-associated protein kinase 70
β-LG	β-Lactoglobulin

Sumário

Várias doenças causadas pelo sistema imunitário, como doenças autoimunes, alergias ou rejeição de transplantes, são causadas por uma resposta inadequada a alguns antigénios. Uma possível forma de tratar estas doenças consiste na indução de tolerância específica e robusta. A tolerância imunitária é definida como a ausência de resposta por parte do sistema imunitário a um dado antigénio, mantendo-se competente para responder a antigénios distintos. Existem vários mecanismos que asseguram tolerância imunitária a antigénios do próprio e vários antigénios ambientais, tais como a selecção negativa no timo de linfócitos autorreactivos juntamente com o desenvolvimento de células T reguladoras (Treg) que suprimem autorreactividade; locais imunoprivilegiados como a placenta; citoquinas imunorreguladoras como a IL-10; e o controlo na periferia de linfócitos maturos através de apoptose, anergia, competição e diferenciação de diferentes populações celulares reguladoras. Nos últimos vinte anos tem sido dada muita ênfase a uma população de células T reguladoras caracterizada pela elevada expressão de CD25 (a cadeia alfa do receptor de IL-2) e de Foxp3 (um factor de transcrição). Com a aquisição de informação sobre as Tregs, os imunologistas têm tido a capacidade de desenvolver novas estratégias para diferenciar e expandir esta população celular, sendo inclusivamente utilizadas Tregs como terapia celular em ensaios clínicos.

Uma forma eficaz de induzir tolerância imunitária em modelos animais consiste na utilização de anticorpos monoclonais (MAbs), dirigidos a

moléculas envolvidas na activação de linfócitos. Vários estudos em modelos animais de patologias do sistema imunitário demonstram a eficácia destes MAbs na indução de uma tolerância robusta a longo prazo. O anti-CD4 não depletante, em particular, é bastante eficaz na indução de tolerância imunitária em transplantação e autoimunidade induzindo uma população de Tregs.

No trabalho descrito nesta tese demonstrámos que o anti-CD4 não depletante é capaz de induzir tolerância num modelo animal de asma alérgica, que é uma patologia dependente de uma resposta imunitária do tipo Th2. O bloqueio do CD4 concomitante com a exposição ao alergénio leva à indução de um estado de tolerância específica para esse antigénio, mantendo-se a competência do sistema imunitário para responder a outros antigénios diferentes. Com base nestes resultados, onde para a indução de tolerância utilizámos antigénios administrados juntamente com um adjuvante (alum), decidimos estudar a indução de tolerância a outras proteínas. Com efeito, estudos prévios mostraram ser particularmente difícil induzir tolerância a factor VIII (FVIII) em modelos animais de hemofilia, utilizando anti-CD4 ou outros anticorpos indutores de tolerância. Decidimos reavaliar a indução de tolerância a FVIII em hemofilia com base na hipótese que um adjuvante poderia facilitar esse processo. Com efeito verificámos que a administração de FVIII com alum permite a obtenção de tolerância robusta ao factor de coagulação com anti-CD4. Estes resultados poderão parecer contra-intuitivos pois os adjuvantes são conhecidos pela sua capacidade de potenciar respostas imunitárias e não de as inibir. No entanto é provável que para indução terapêutica de tolerância seja necessário que o antigénio a tolerar seja apresentado de forma eficaz à maioria de células T efectoras específicas durante o período

em que o anti-CD4 se mantém em níveis terapêuticos. Um adjuvante poderá assim facilitar a apresentação de antigénios pelas células dendríticas durante este período. Esta hipótese será avaliada em experiências futuras.

Finalmente, procurámos identificar os mecanismos celulares e moleculares associados à indução de tolerância imunitária a proteínas em adjuvante. Como está descrito na literatura que o anti-CD4 promove, em transplantação e autoimunidade, a diferenciação de Tregs Foxp3^{+}, assumimos que o mesmo mecanismo estaria envolvido na indução de tolerância a proteínas em alum. Contudo, verificámos ser possível indução de tolerância em animais deficientes em Foxp3. No entanto o estado de tolerância depende de IL-10, pois não é possível induzir tolerância em animais na ausência desta citoquina. Para além deste mecanismo de tolerância dominante dependente de IL-10 também está envolvido um componente recessivo, que depende da anergia e apoptose de células efectoras específicas

É possível que a combinação de estratégias que promovem mecanismos indutores de tolerância independentes de Foxp3 e outras estratégias que promovam tolerância mediada por Treg possam ser explorados de um modo sinergético para alcançar um estado de tolerância mais robusto e em situações mais estringentes.

Abstract

Several immune mediated diseases, such as autoimmunity and allergy or transplant rejection are consequence of inappropriate immune responses towards specific antigens. The therapeutic induction of robust immune tolerance has, therefore been considered the Holy Grail of immunology since the pioneering work of Medawar and colleagues who have demonstrated the feasibility of tolerance induction. Immune tolerance is defined as the unresponsive state to a set of antigens by an immune system that remains immune competent to respond to unrelated antigens. A wealth of mechanisms has evolved to achieve immune tolerance to self as well as many environmental antigens. These mechanisms include negative selection in the thymus together with the production of regulatory T cells dedicated to the prevention of auto reactivity; cytokines like IL-10; immune privileged sites; and peripheral control of effector T cells by apoptosis, anergy, competition, and differentiation of several types of regulatory immune cells. In the last twenty years much emphasis has been given to a powerful regulatory T (Treg) cell population initially characterized by the high expression of CD25 (the alpha chain of the IL-2R), that express the transcription factor Foxp3. Over the years a significant amount of information on Tregs has been acquired which empowered the immunologists to devise new strategies to differentiate and expand these cells and ultimately to use them as cellular therapies.

One strategy known to induce immune tolerance takes advantage of biological agents such as monoclonal antibodies (MAbs) that can specifically target certain molecules modulating immune responses. Several studies in different animal models of immune disorders have shown the efficacy of MAbs at inducing long term specific immune tolerance. Non depleting anti-CD4 MAb has been shown able to induce immune tolerance in transplantation and autoimmunity by inducing a specific Treg population.

In the work presented in this thesis we have shown that non depleting anti-CD4 can induce robust tolerance to soluble antigens in a mouse model of allergic asthma – a Th2-driven pathology. CD4-blockade at the time of allergen exposure can lead to antigen-specific tolerance therefore sparing the immune response to other antigens.

Based on these findings, where the allergen is administered together with an adjuvant, we revisited immune tolerance to therapeutic proteins using a mouse model of hemophilia. It had been reported that tolerance induced with MAbs (such as anti-CD4) was difficult to induce to proteins like factor VIII. We hypothesized that an adjuvant, such as alum, that in the animal models of allergy allows tolerance induction to model antigens and allergens, may be useful to facilitate tolerance to FVIII. We confirmed that indeed tolerance induction to FVIII can be induced when alum is used as an adjuvant – a counter-intuitive finding given the tendency of adjuvants to boost, rather than inhibit, immune responses. We believe that for proper tolerization of antigen-specific cells it is essential to have adequate antigen presentation. Therefore an adjuvant can facilitate the process. This hypothesis will be addressed in future studies.

Finally, we investigated the cellular and molecular mechanisms underlying the induction of tolerance to proteins using non-depleting anti-CD4. We anticipated that induction of Foxp3+ T cells would be a major mechanism given prior studies that have clearly established induced $Foxp3^+$ Tregs in tolerance induction in transplantation and autoimmunity. However, we found that tolerance to proteins in alum could be induced in Foxp3-deficient mice, but not in the absence of IL-10. In addition to the dominant tolerance mechanism IL-10-dependent we found evidence for a recessive mechanism based on the activation-cell death and anergy of antigen-specific cells following activation under the cover of anti-CD4.

We believe it may be possible to take advantage of both Foxp3-independent and Foxp3-dependent mechanisms to achieve a more robust state of immune tolerance.

Introduction

For many decades self/non-self discrimination by the immune system was assumed to be a consequence of clonal selection of effector T cells. Although the concept of *horror autotoxicus* was postulated by Ehrlich over a century ago, the first model proposing the elimination of self-reactive T cells during thymic development was only proposed in 1959 [1]. This clonal selection theory is still regarded as the major mechanism underlying central tolerance: the events in the thymus during early T cell differentiation that lead to the deletion of potentially self-reactive T cells.

In the thymus cortex $CD4^+CD8^+$ (DP) cells originating from $CD4^-CD8^-$ (DN) T cell precursors [2] establish their first ligations with MHC molecules. Failure to engage an MHC within 3 to 4 days results in death for these T cell precursors [3]. This event is called “death by neglect” and eliminates around 95% of these cells as they fail to receive essential survival signals [3]. The remaining 5% cells survive by engaging the MHC of cortical thymic epithelial cells (cTECs) loaded with intracellular protein fragments [4] thus receiving a survival signal that allows positive selection [5]. Defects in the ZAP-70 signaling can alter the T cell repertoire and favor autoimmunity by allowing the survival of autoreactive cells that would otherwise be deleted [6-8]. Upon positive selection T cells become single positive for CD4 or CD8 [9] and in the medulla suffer further refinement of their specificity by engaging MHC molecules on medullary thymic epithelial cells (mTECs) and dendritic cells in a process called negative selection [10]. In the medulla mTECs expressing high levels of both CD80 and MHC class II are also positive for Aire [11]. Aire directs expression of self-antigens within the thymus preventing autoimmunity by negative selection of cognate T cells [12, 13] in a dose dependent fashion [14].

However clonal selection does not explain the entire scope of immune tolerance. It became apparent that dominant cellular immune regulatory mechanisms, exerted by T cells, have a critical role in suppressing autoimmune targeting by peripheral T cells [15]. This issue has been controversial until a definite characterization of the phenotypic and functional characteristics of this population of T cells dedicated to tolerance maintenance was established [16]. Environmental cytokines and co-stimulatory factors may favor a functionally unique differentiation program for T cells that experience strong TCR signaling leading to a selection by agonist interaction of Treg cells with a repertoire skewed towards self-reactivity [17]. In the thymic development of natural Tregs (nTreg) CD28 plays a key role, as deficiency in CD28 or its ligands B7-1/B7-2 lead to decreased thymic nTreg cells [18-20]. CD28 signaling is provided by thymic APCs [21] which might suggest that specialized APCs can be responsible for the different T cell populations selected by agonist. Downstream of CD27/B7 pathway, c-Rel a member of the NFκB family is a crucial factor for nTreg development in the thymus [22]. Mice deficient in c-Rel have shown a reduction in $Foxp3^{+}$CD4SP cells in the thymus and in the periphery [23], while NF-κB1 had no effect [23]. In addition to co-stimulation, cytokine signaling is also of major importance, especially IL-2. Nevertheless IL-2 might have a redundant effect with other common γ-chain cytokines like IL-15 as the lack of IL-2 is less significant in reducing nTregs than the lack of its receptor IL-2Rβ [24-26]. Thymic Treg development appears to follow a two-step instructive model: TCR engagement leads to upregulation of CD25 (IL-2Rα) and other components of the proximal IL-2 signaling pathway and, subsequently, to IL-2-dependent expression of Foxp3 [27]. Curiously STAT5 constitutive expression deflects thymocytes normally destined to become naive T cells

into the Treg cell lineage [28] which further supports the crucial role of IL-2 in nTreg development.

Treg cells are characterized by expression of the forkhead-family transcription factor Foxp3, whose function is indispensable for their development, maintenance and function [29]. Nr4a triple KO mice to show that Nr4a receptors have redundant yet crucial roles in thymic Treg cell development [30] acting as effectors of thymic CD4+ T cell fate by 'translating' the strength of TCR signaling [30]. Epigenetic studies show that nTregs have a stable Foxp3 expression due to the demethylation of CpG motif in the Foxp3 locus [31].

While central tolerance ensures that most T cell that exit to the periphery do not recognize self-antigens, peripheral tolerance ensures a cellular mechanism, mediated by regulatory cells, that self-antigens effector T cells that eventually escape to the periphery recognizing are suppressed and do not lead to autoimmune pathology.

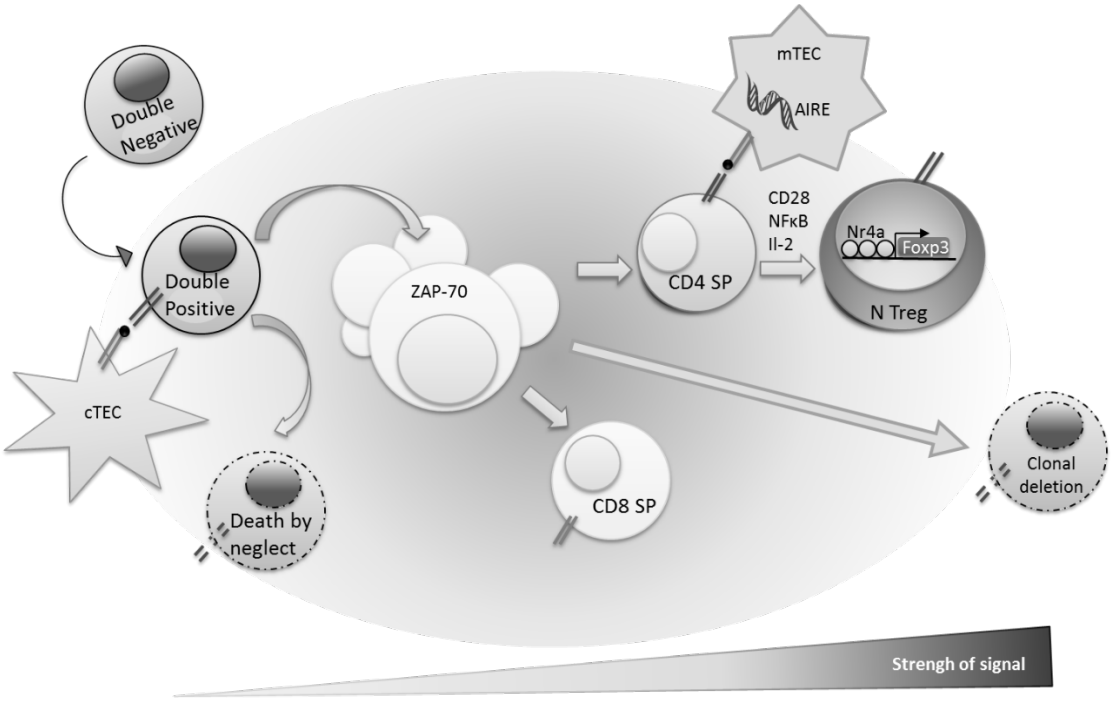


Figure 1. The reactivity of the TCR for self-MHC ligands defines the outcome of the DP T cells. Cells with no ability to react with thymic ligands will die by neglect. Those with low affinity are positively selected and become $CD4^+$ or $CD8^+$ T cells. Those with too high affinity undergo clonal deletion to preserve self-tolerance. Those cells that receive intermediate TCR signaling in the thymus suffer an agonist selection and acquire a special phenotype with distinct function.

Recently it is becoming apparent that the importance of dominant regulation goes beyond the discrimination of self and non-self: it also discriminates between harmful and innocuous. In fact, cellular mechanisms, as detailed below, persistently patrol the organism preventing the onset of inflammation, namely allergic inflammation. The biological significance of this active tolerance-imposing mechanism is well demonstrated by the severity of the allergic and autoimmune syndrome that arises in individuals that lack this ability to tolerate self and harmless antigens.

Indeed, the organism is constantly exposed to non-pathogenic antigens that, in healthy individuals, are tolerated. It is, however, common (and becoming increasingly frequent) that an overzealous immune system will activate and develop effector responses to such harmless antigens developing allergy and other inflammatory diseases. Over the last decades allergic diseases, including allergic asthma, atopic dermatitis and food allergy, have become a major health problem in developed countries [32]. Despite the advances in the understanding of the pathophysiology of allergy and in its clinical management, allergic pathology remains a significant burden on the quality of life and economy of western society. Several strategies have been devised to overcome the pathological immune response by inducing immune tolerance.

A major cellular mechanism in maintaining immune tolerance is the population of natural (or thymic derived) Foxp3$^+$ Treg cells [33, 34]. Indeed these have been clearly implicated as potent inducers of a non-responsive state in several immune mediated pathologies like autoimmunity, transplantation, graft-versus-host disease, and allergy [35-39]. It has been shown, in allergy, that regulatory T cells can be transferred conferring specific tolerance to subsequent challenges with the allergen [40, 41]. In addition, depletion of the regulatory T cells can have a detrimental effect in allergic airway hyperreactivity [42]. Importantly Foxp3-deficiency, in mice and human beings, leads to a severe immune deregulation syndrome characterized by allergic and autoimmune manifestations that are rapidly fatal [43]. In addition to the important role of natural Foxp3$^+$ Treg cells (nTreg) in preventing autoimmunity, it has become established that Foxp3 expression can be peripherally induced following T cell activation in presence of TGF-β [44]. These peripherally

induced Treg cells (iTreg) are believed to be important for tolerance induction to non-self-antigens, including allergens [44].

Induction of regulatory T cells

The study of peripheral induction of Treg cells was greatly facilitated with the use of *Rag*-insufficient TCR-transgenic mice, with the TCR specific for a non-self antigen. In these mice nTregs cannot be formed in the thymus due to the absence of a selecting thymic antigen. In 2003 it was shown that conventional T cells can be converted into iTreg *in vitro* when activated in presence of TGF-β[45]. In addition those iTreg cells were fully capable of controlling airway hyper reactivity (AHR) in previously sensitized mice [45-49]. It was subsequently found that reducing or blocking the available amount of TGF-β exacerbates AHR [50, 51], while the local delivery of this cytokine or adoptive transfer of T cells engineered to express latent TGF-β rescue mice from antigen sensitization and therefore prevent AHR[52, 53]. Interestingly, sub-optimal TCR signaling together with TGF-β greatly enhances iTreg conversion [54], which is in agreement with *in vivo* data showing that repeated low doses of allergen exposure promotes the emergence of $Foxp3^+$ iTregs expressing TGF-β on the membrane[55]. Under sub-optimal TCR stimulation, obtained with a low-dose of plate bound anti-CD3 or with DCs pulsed with a low dose of agonist peptide, as well as, with down-modulation of the TCR with non-depleting anti-CD4, promotes iTreg conversion in the absence of exogenous TGF-β [56]. Under those conditions Foxp3 expression still requires TGF-β, but the T cells can produce TGF-β, and benefit from the presence of this cytokine for conversion to Treg [56].

In addition to the importance of TGF-β for iTreg conversion, some studies showed that TGF-β can directly inhibit GATA3 expression thus impairing Th2 differentiation [57-59]. Because the Th2 response is impaired, the production of IL-4 is diminished, and this has a direct impact on B cell class switch preventing IgE and favoring IgA production [60].

It is also becoming apparent that the environment influences the outcome of T cell activation, and the decision to induce Foxp3 and regulatory properties. Several reports have shown that the mucosal surfaces have a role in establishing an iTreg population: alveolar epithelial cells have been reported to participate in iTreg induction in a mechanism dependent of MHC class II expression and TGF-β [61]. Both alveolar and gut epithelia have been shown to depend on retinoic acid together with TGF-β to induce tolerance [62, 63]. It was also found that retinoic acid in the presence of TGF-β impaired STAT6 binding to the Foxp3 promoter therefore enhancing histone acetylation and reverting the repressive the effect of IL-4 on the Foxp3 promoter[64].

Despite the critical role of TGF-β in iTreg induction and henceforth tolerance, this cytokine can also have some adverse effects since it is instrumental in the differentiation of Th17 and Th9 together with IL-6 and IL-4, respectively [65, 66]. Note that although Treg cells can prevent allergic autoimmune encephalomyelitis (EAE), a disease promoted by Th17 cells, mice with T cells with a dominant negative receptor for TGF-β1 do not develop EAE as Th17 cells are not induced [67]. Moreover, TGF-β has also been implicated in tissue remodeling, by induction of collagen expression in fibroblasts, as well as goblet cell proliferation and mucus production [68].

Regulatory T cells and IL-10

Although TGF-β is the major known driver of iTreg differentiation, IL-10 has been shown to be another key player that has been vastly described in protection from allergic diseases [69].

Studies with bee venom specific immunotherapy have shown that tolerance to the allergen can be induced in a process that is IL-10-mediated [70]. In addition, respiratory exposure tolerance induction to OVA relied on antigen specific $CD4^+$regulatory cells that produced IL-10 [71]. Tolerance was transferrable and abrogated when IL-10 or ICOS ligand was blocked. Interestingly, those regulatory cells shared some features with effector Th2 cells: both populations expressed IL-4 and IL-10 although in different amounts. While the regulatory cells primarily release IL-10, the effectors rely on IL-4 as the main cytokine. It has been suggested that different types of effector cells, including Th2, produce IL-10 at the end of the immune response in a mechanism that is important in limiting their inflammatory behavior [72]. IL-10 producing T cells have been described able to control the late response in allergic asthma by reducing neutrophilia [73]. It has been suggested that Foxp3-negative IL-10 producing T cells, can be induced following activation in presence of IL_10, and constitute a population of regulatory cells different from Foxp3+ Tregs that are named TR1 [74, 75]. TR1 cells have been identified in mice and humans, and there are currently clinical trials [39, 76]. There are several other lines of evidence demonstrating the crucial role of IL-10 in the prevention of airway inflammation: IL-10-deficient mice have an exacerbated allergic airways response with high levels of pro-inflammatory cytokines like IL-5 and IFN-γ in the BAL [77]. Furthermore, intranasal administration of rmIL-10, concurrently with OVA, inhibited both airway neutrophilia and

eosinophilia [78]. It was also shown that allergen-specific $CD4^+CD25^+$ Tregs can suppress allergic airway disease in vivo through an IL-10-dependent mechanism [48]. In this study, adoptive transfer of Treg cells reduced AHR, Th2 and eosinophil recruitment into the airways, and secretion of Th2-type cytokines. The effect was IL-10 mediated, since neutralizing anti–IL-10R abrogated suppression. In addition, these effects were independent of IL-10 production by the $CD4^+CD25^+$ regulatory cells themselves [48].

Unlike TGF-β, IL-10 does not directly influence B cell class switch [79]. However it is possible that indirectly, by inhibiting the inflammatory response, IL-10 shapes the humoral outcome. Indeed, it was proposed that IL-10 may favor the ratio of IgG4/IgE ratio [80]. In fact immunotherapy studies show that Th2 responses can be suppressed by IL-10 secreting regulatory cells accompanied by an increase of circulating IgG4 [81, 82].

Different subsets of regulatory T cells

$Foxp3^+$ Treg cells, despite an apparent phenotypic uniformity and immunosuppressive function, can have different subtypes with distinct genetic signatures. The first major division was identified between nTreg and iTreg, where the first are enriched in Helios, a transcription factor that is primarily expressed in T-lineage cells and early precursors [83, 84]. While nTreg cells have epigenetic mechanisms that stabilize Foxp3 expression allowing them to be a stable differentiated cell lineage, TGF-β induced Tregs lack those mechanisms having incomplete demethylation of the Foxp3 gene [31]. Therefore, although iTreg cells have high levels of Foxp3, the expression of Foxp3 is less stable [31, 85, 86]. In addition, conserved non-coding DNA sequence (CNS) elements at the Foxp3 locus encode information defining the size, composition and stability of the Treg cell

population [87]. CNS3, which binds c-Rel, has a drastic effect on the frequency of Treg cells generated in the thymus. Contrary to CNS3, CNS1 has no effect on thymic generation of Treg cells but is essential for induction of iTregs [87]. CNS1 contains a TGF-β–NFAT response element, so these results could represent the requirement of TGF-β and NFAT for Treg induction in the periphery [87-89]. Although CNS2-deficient T cells can acquire Foxp3 expression, they fail to maintain Foxp3 expression on their progeny due to the failure on recruitment of Foxp3–Runx1–Cbf-β complexes to CNS2 after demethylation of the CNS2 CpG island [87, 90]. Interestingly CNS1 deficient mice had no lymphoproliferative disorder. However, it can be argued that these animals kept in clean facilities have a minimal exposure to foreign antigens and thus nTreg may be sufficient to maintain homeostasis in such conditions. In effector T cells, GATA-3 is a hallmark of the Th2 cells, but Treg cells can also express GATA-3, that binds both to the Th2 cell locus and to the CNS2 of Foxp3 locus [91]. In fact, there is a dramatic increase of GATA-3 binding to CNS2 compared to conventional T cells, suggesting that GATA-3 regulates CNS2 activity in Treg cells [91].

There is strong evidence that the CCR7-dependent continuous migration of DC from the lung to its draining LNs is required for the transport of inhaled Ag and thereby for the proper composition of APCs in the LN. These processes are essential to induce peripheral tolerance of T cells [92]. The co-stimulation with ICOS, crucial for regulatory phenotype polarization in allergy[93], promotes the down-regulation of CCR7 and CD62L after activation, leading to a reduced return of activated CD4 T cells to the lymph nodes and a more efficient entry into the lungs[94]. Regulatory T cells express CCR4 and CD103 induced by antigen-driven activation in the lymph nodes. In addition, the accumulation of Tregs in the

skin and lung airways is impaired in the absence of CCR4 expression [95]. Mice without CCR4 in the Treg compartment develop lymphocytic infiltration and severe inflammatory disease in the skin and lungs [95]. Some studies suggest that CCR4 has a prominent role in effector Th2 homing [96]. Despite their differences it seems both regulatory and effector T cells share the response to homing factors [97, 98].

But GATA-3 is not the only transcription factor characteristic of effector T cell responses that can be expressed by Foxp3$^+$ Treg cells. Under the influence of IFN-γ, Foxp3$^+$ Treg cells can express the Th1-defining transcription factor T-bet [99]. T-bet expression by Foxp3$^+$ Treg cells induces the expression of the chemokine receptor CXCR3, necessary for these Treg cells to accumulate at the site of type-1 inflammation. T-bet expression was thus required for the homeostasis and function of Treg cells during type-1 inflammation [99].

It is likely that the regulation of different types of immune response require the participation of specialized subsets of regulatory cells. This way, iTreg cells induced in an environment favorable to Th1 or Th2 type of immune responses, require the appropriate chemokine receptors to give them access to the same locations as effector T cells (Figure 2).

Th17 cells that have been implicated in autoimmunity and allergy share with iTreg cells the need for TGF-β to differentiate [100]. The decision of antigen-stimulated cells to differentiate into either Th17 or iTreg depends on the cytokine balance of IL-6, IL-21 and IL-23 that relieve Foxp3-mediated inhibition of RORγt [101]. These results indicate that Foxp3 and RORγt are transcription factors that antagonize each other in the lineage differentiation.

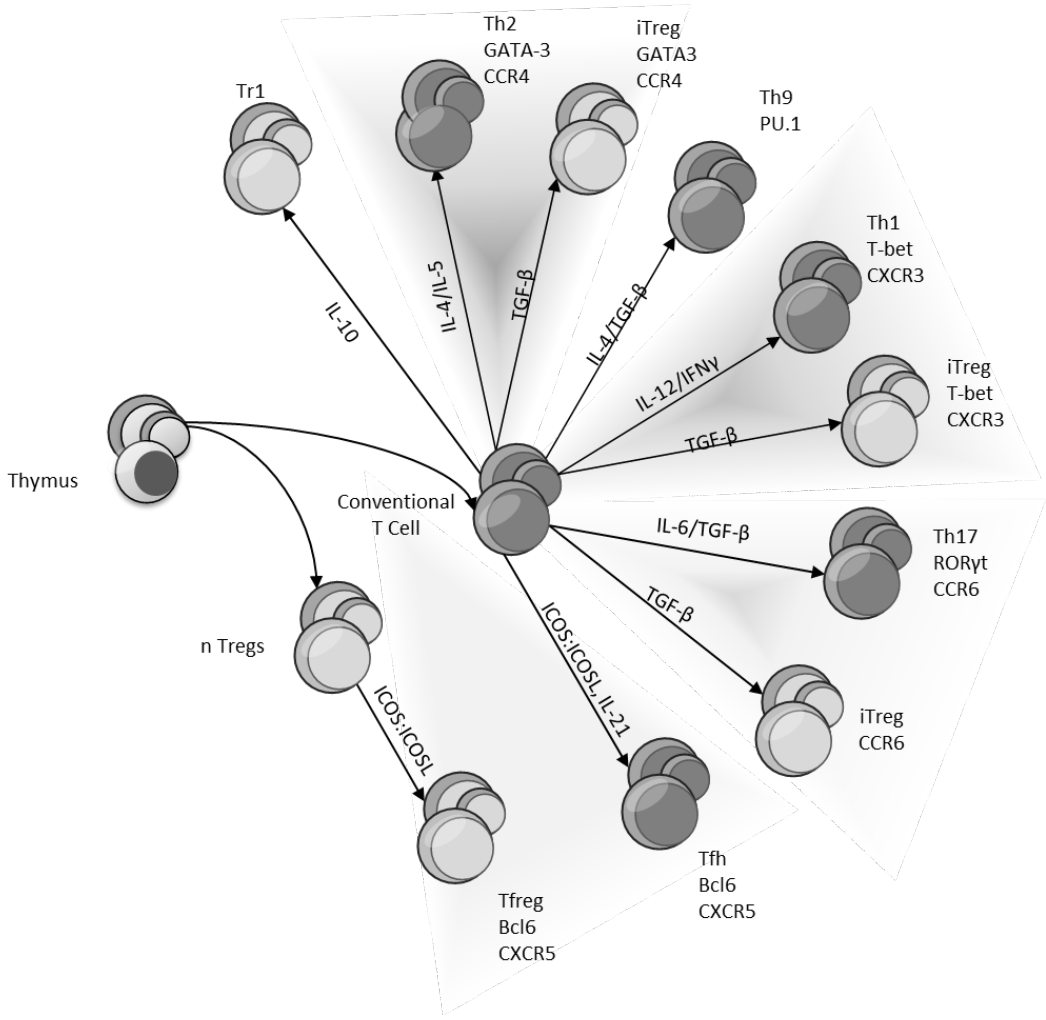


Figure 2. Differentiation of effector and regulatory T cells under the influence of different cytokines.

Different types of immune responses carry different cytokine microenvironments that can influence both effector and regulatory T cell differentiation. Like the effector T cells, regulatory T cells can also display different molecules on their surface that will allow them to migrate to the inflammation site. Although this seems to be a feature mostly associated with iTregs, the Tfregs, whose precursors are nTregs, also change their chemokine receptor profile in order to enter the follicles and exert their regulatory functions.

Another subset of T cells, the follicular T helper cells (Tfh), is mostly spatially confined to secondary lymphoid organs, more precisely to the B cell follicles [102]. Tfh cells express high levels of the transcription factor

Bcl-6, that impairs the expression and function of other transcription factors specific for other CD4 subsets: Tbet, GATA3, and RORγt, thereby regulating cytokine production by Tfh cells [103, 104]. Tfh cells differentiate under the influence of ICOS: ICOSL and IL-21 but independently of any other cytokine [105]. In addition, the characteristic anatomical distribution of Tfh cells is dependent of CXCR5 that endows access to the B cell follicle [102, 106, 107]. It has been recently found that also this subset of effector T cells has a specialized regulatory counterpart [98, 108, 109]. It was found that Foxp3+ Treg cells can be found within the B cell follicle [110], sharing many characteristics of Tfh and Treg cells [98, 108, 109]. Importantly,

Bcl-6 can be co-expressed with Foxp3 as it seems Foxp3 expression is not inhibited by Bcl-6. These follicular regulatory T cells (Tfr) are immune-suppressive and can control de magnitude of the germinal center response [98, 108, 109]. In addition, they exhibit a $CTLA4^{hi}GITR^{hi}IL\text{-}10^{hi}$ phenotype that is characteristic of activated Tregs [98, 108, 109]. However, the Tfr origin is quite distinct from the other induced Treg cells previously described. Tfr cells do not derive from the commitment of conventional CD4 T cells, but result from acquisition of “follicular” characteristics (namely Bcl-6 expression) by natural Foxp3+ Treg cells[98, 108, 109]. In fact sorted Tfh cells exposed to optimal conditions to induce Foxp3 expression in conventional T cells (including TGF-β) resist conversion to Tfr [108] . Given the importance of the germinal center response for allergy, it is likely that Tfr cells can play an important role in regulating IgE production.

Besides conventional T cells, also natural killer T (NKT) cells are important players in defining the outcome of immune responses. Notably, invariant NKT (iNKT) cells were found able to help B cell differentiation, germinal-center formation, affinity maturation and immunoglobulin

response that was uniquely dependent on iNKT cell–derived IL-21, although the GCs maintain a small size throughout the reaction [111, 112]. This contribution of iNKT cells for humoral responses can be added to their ability to contribute to allergic airways diseases by producing IL-4 and IL-13 [113, 114], or IL-17[115, 116]. But iNKT cells can also have a regulatory role, namely in preventing

EAE following administration of its TCR agonist [117, 118]. We and other recently described that activation of murine or human iNKT cells in presence of TGF-β induces Foxp3 expression and acquisition of suppressive function [117, 119].

Influencing regulatory T cells in allergy

The understanding of the mechanisms involved in regulatory T cells generation and function may lead to novel strategies to restore immune tolerance where it has been lost. As TGF-β and IL-10 play a crucial role in tolerance induction, several studies on immune tolerance induction took advantage of environments rich in those anti-inflammatory cytokines. To our advantage the mucosa itself is an anatomical location rich in these immune mediators [120].

Airborne antigens can be transferred from the mother to the newborn through milk [121]. Breastfeeding-induced tolerance was found to be mediated by induced $Foxp3^+$ Treg cells and dependent on TGF-β [121]. On the other hand it has been proposed that metallo-matrix proteases, derived from commensal bacteria in the gut, can facilitate the conversion of latent TGF-β to its active form, thus favoring iTreg differentiation [122]. In addition, $CD103^+$ dendritic cells in the mucosa-

draining lymph nodes have been shown effective in promoting conversion of iTregs in the gut, mediated by TGF-β and the synthesis of retinoic acid, a powerful inducer of Foxp3 expression [62, 123, 124]. Furthermore, vitamin D receptor deficient mice were associated with a reduction in tolerogenic $CD103^+$ dendritic cells favoring the development of effector type T cells [125]. Vitamin D3 can be used to induce human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into regulatory cells that produced only IL-10, but no IL-5 and IFN-γ, and furthermore retain strong proliferative capacity [126]. Several other studies put vitamin D3 in relevance as acting directly on T cells to induce IL-10^+ regulatory cells and also influencing levels of TGF-β [127-129]. These data suggest that the mucosa, in particular the gut, has several mechanisms that can favor immune tolerance. Sublingual immunotherapy (SLIT) and oral immunotherapy (OIT) are becoming more relevant as effective tolerance-inducing strategies to treat inhalant as well as food allergies [130].

Allergen specific immunotherapy (SIT) which comprehends of SLIT, OIT and subcutaneous immunotherapy (SCIT) has been in clinical use for around 100 years [131] and consists on the administration of increasing doses of an allergen [132]. It has been shown that both $Foxp3^+$ and IL-10 positive regulatory T cells can be induced during the course of SIT protocols [133, 134]. Furthermore, allergen-specific TR1 cells, in healthy individuals, have been suggested to play a key role in preventing pathologic responses [82, 131, 135]. While the presence of IL-10 leads B cells to produce IgG4 in detriment of IgE [136, 137], TGF-β drives B cells to switch to IgA production [135]. Another approach to direct the organism towards a tolerant state arises from the results that suggest that reduced TCR stimulation favors the induction of a regulatory phenotype on the T cells [56, 138, 139]. Blockade of molecules involved in the immune synapse

has been suggested as an approach to achieve suboptimal TCR activation [56, 139]. Blockade of CD4 was shown a robust approach to achieve Treg-mediated dominant tolerance in transplantation [140-142]. The realization that active regulatory mechanisms, such as the ones mediated by Treg cells, can prevent pathological immune responses to harmless antigens is changing the way immunotherapy is perceived. In very diverse fields of immunology, ranging from cancer immunotherapy to autoimmunity and allergy, regulatory mechanisms need to be considered when therapeutic interventions are designed to boost or dampen the immune response. The realization that different subsets of regulatory T cells exist may offer the possibility to fine tune such interventions in order to achieve optimal therapeutic benefit with limited immunosuppressive consequences in unrelated immune responses.

At a time when therapeutic interventions rely increasingly in potentially immunogenic drugs – such as recombinant proteins to correct genetic diseases or monoclonal antibodies, where even the human antibodies can be immunogenic due to their unique idiotypes [143, 144], the issue of tolerance induction to non-self antigens will not be restricted to allergy and transplantation, but a growing concern for drug efficacy.

Induction of Dominant Tolerance Using Monoclonal Antibodies

Ever since the description of classical transplantation tolerance by Medawar and colleagues [145], the attainment of clinical transplantation tolerance has been considered the “Holy Grail” of immunology. Two decades have passed since the initial demonstration that long-term

transplantation tolerance can be induced following a brief treatment with monoclonal antibodies (MAb)[146-148] and that tolerogenic anti-CD4 MAb can be used to treat EAE [149]. However, the mechanisms by which tolerance is induced and maintained are not yet fully understood. In recent years it has become clear that antibody induced transplantation tolerance leads to both deletion of some alloreactive clones and regulatory T cells cell expansion [38, 150].

In the first examples of peripheral tolerance induced with MAb, depleting anti-CD4 MAb was used to induce tolerance in mice to foreign immunoglobulins [146, 147]. It was soon demonstrated that depletion of CD4+ T cells was not required for tolerance induction, as similar results were obtained using Fab'2 fragments [151, 152], non-depleting isotypes [153]or non-depleting doses of synergistic pairs of anti-CD4 MAb [154]. In other studies a brief treatment with anti-CD4 MAb was also shown to lead to long-term acceptance of skin grafts differing in multiple minor antigens [153], even in pressensitized recipients [155]. The same results were also demonstrated for heart grafts across major histocompatibility complex (MHC) barriers [156, 157] or concordant xenografts [156]. The treated animals accepted the transplanted tissues indefinitely without the need for immunosuppression, and remained fully competent to reject unrelated (third-party) grafts. Clearly the antibody treatment had rendered them tolerant to antigens belonging to the transplanted tissue (see Fig. 3).

Figure 3. Induction of transplantation tolerance with monoclonal antibody (MAb). Mice transplanted under the cover of tolerogenic MAb accept skin grafts indefinitely. To demonstrate that mice are indeed tolerant, one can simultaneously transplant a new graft of the tolerized type and graft an unrelated third-party donor, showing that tolerant mice remain fully competent to reject latter.

Following these studies, it became clear that MAb, other than anti-CD4, could be used to impose peripheral transplantation tolerance (see Table 2). Tolerance induced with MAb, such as CD4 or CD154 (CD40-Ligand), is dependent on Treg cells both $CD4^+CD25^+$ and $CD4^+CD25^-$ [158]. These Treg cells can be demonstrated both within lymphoid tissue and infiltrating the tolerated transplant [159]. The dominant tolerance state, maintained by the Treg cells, allows the immune system to resist the adoptive transfer of non-tolerant lymphocytes without tolerance break down [153, 160]. In addition, adoptively transferred non-tolerant lymphocytes are rendered tolerant and regulatory when allowed to coexist with the initial cohort of Treg cells—a process usually referred to as infectious tolerance [142, 161, 162]. The dominant tolerance state can be extended through linked suppression to additional antigens, if those new antigens are provided genetically linked with the tolerated ones within a new transplant [141, 161, 163, 164].

In addition to tolerogenic antibodies, several experimental procedures may be used to facilitate tolerance induction.

Such is the case with the administration of donor bone marrow (BM), or donor blood (donor- specific transfusion [DST]). The elimination of cell populations that may create an obstacle to graft survival with weak

tolerogenic antibodies is also commonly used. Such is the case for CD154 tolerogenic antibodies, able to induce dominant tolerance within the CD4+ T cells, but when used alone cannot prevent skin graft rejection mediated by CD8+ T cells [164, 165]. As a consequence, CD8 depletion is often used when the tolerogenic properties of CD154 MAb are studied.

Tolerogenic MAbs have been also used to reprogram the immune system in animal models of autoimmune diseases [166]. However, it has been more difficult to confirm that the tolerant state, so induced, is dominant and mediated by Treg cells than in the context of transplantation, as experiments with third-party antigens are harder to conduct. Nevertheless, the characterization of the phenotype of the dominant Treg population in maintaining self-tolerance as being CD4+CD25+Foxp3+ has permitted the confirmation that tolerogenic MAbs, such as the ones directed toward CD3, can prevent autoimmunity by eliciting Treg cells that maintain the tolerant state in a dominant manner [167]. As a consequence, it appears that MAbs, known to be able to induce dominant transplantation tolerance, may also restore dominant self-tolerance in autoimmunity.

Induction of tolerance in allergic respiratory disorders with MAb.

The advances in the knowledge about the allergic disease have empowered researchers to pursue new therapies within the immunomodulatory agents. The complicated network of cells and

molecules involved in the Th2 response has given several opportunities that are being explored towards the treatment of diseases such as allergic asthma [168, 169].

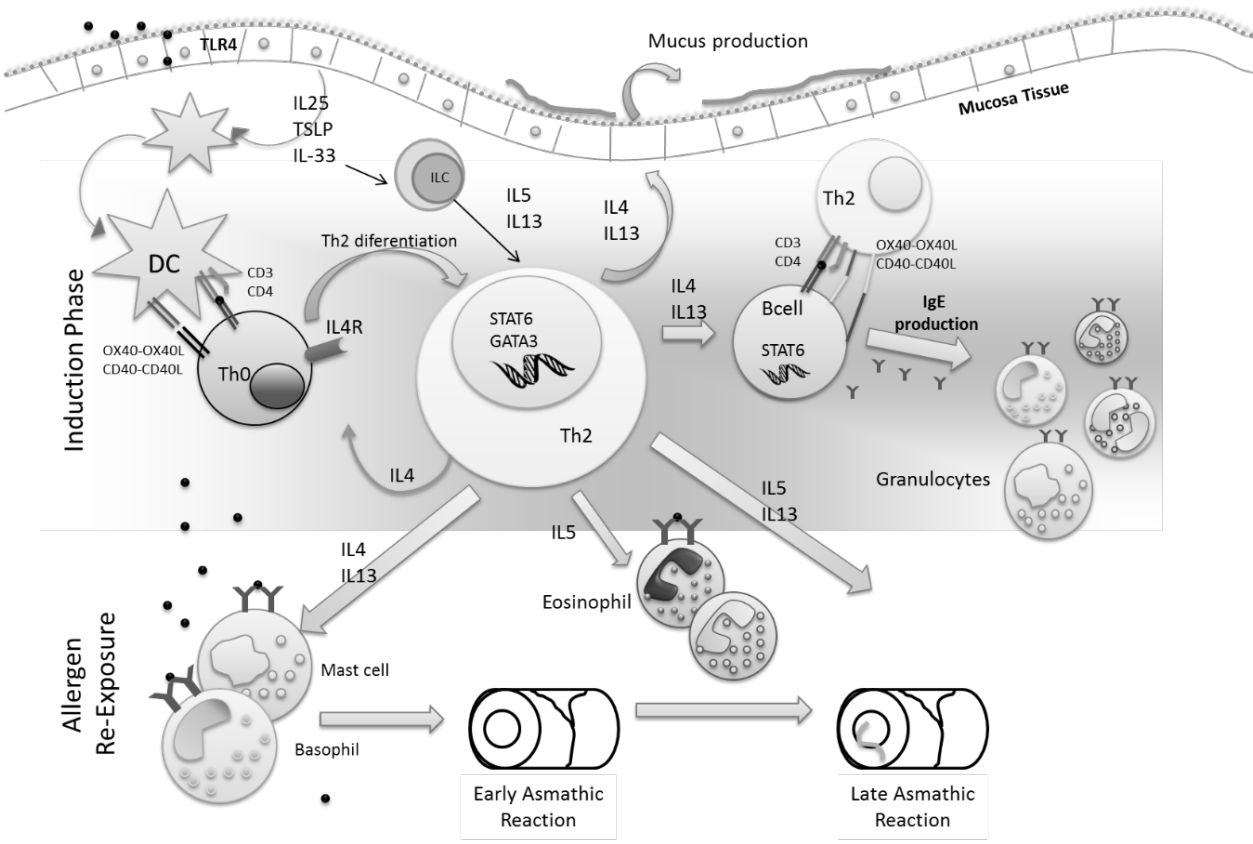


Figure 3. The complex network of the allergic airway sensitization. The antigen recognized by the Toll-like receptor 4 (TLR4) induces the tissue to release cytokines that promote not only dendritic cell (DC) maturation but also T cell differentiation from nuocites (ILC) [170] and recruitment of innate immune cells like mast cells and basophils. The Th2 cell will later recruit more innate cells like the eosinophils by the release of IL-5, and promote B cell differentiation into a plasma cell that releases immunoglobulins that potentiate and perpetuate the disease. IgE and IgG_1 can bind and activate mast cells and basophils by means of the FcξRI [171-173] triggering the release of histamine that can affect several cell types in immune and non-immune systems [174].

Most strategies rely on either blockade of cytokines or blockade of co-activators and co-stimulators molecules (see table 1). The majority of the research developed has had positive results and is now in clinical trials [169]. There is little evidence however in the literature on how these therapies affect the regulatory T cell population. The IL2:Anti-IL-2 MAb is described to promote an IL-10 secreting $Foxp3^+$ T cell population that effectively induces tolerance in allergic airway disease [175]. We recently shown that a non-depleting anti-CD4 monoclonal antibody can induce in mice robust, antigen-specific tolerance to house dust mite, even in pre-sensitized animals [46]. In addition, a similar strategy was effective to prevent peanut-induced anaphylaxis in mice [176]. Co-stimulation blockade was also shown to be effective in preventing allergic sensitization in mice. Regarding the different modalities for co-stimulation blockade, on one hand CTLA4Ig was shown able to greatly reduce the secretion of IL-4 but not enough to impair Th2 response [177]. On the other hand, treatment with OX40L-blocking MAbs inhibited to some extent allergic immune responses induced by TSLP in the lung and skin, preventing Th2 inflammatory cell infiltration, cytokine secretion and IgE production in mice and nonhuman primate models of asthma [178]. [179]. Based on previous studies of tolerance induction to alloantigens following co-stimulation blockade, it is likely the mechanism also relies on Treg cells [162, 180].

Table 1. Immunomodulatory MAbs used in allergic pathologies

Antibody Target	Effect	Reference
IL-2:anti-IL2	Prophylactic and tolerogenic	[175]
IL-4	Is prophylactic in AHR but no effect in eosinophilia	[181, 182]
IL-5	Anti-inflammatory	[182, 183]
IL-13	Prevents progression in established disease	[184, 185]
IL-25	Prophylactic and anti-inflammatory	[186]
IL-33	Anti-inflammatory	[187]
TSLP	Anti-inflammatory	[188]
LFA-1	Prophylactic	[189, 190]
CD4	Prophylactic and prevents progression of established disease	[46]
CD23	Anti-inflammatory	[191, 192]
OX40L	Prophylactic	[178]
CD40	Has a prophylactic role when administered together with CTLA4Ig	[179]
CTLA4Ig	Together with anti-CD40L is prophylactic	[177]
CD147	Anti-inflammatory	[193]
CCR3	Ablate eosinophilia in established AHR	[194]
TIM-1	Anti-inflammatory	[195]
TIM-3	Prophylactic	[196]
TNF	Prophylactic	[197]
4-1BB/4-1BBL	Prophylactic and anti-inflammatory	[198-200]
IgE	Prophylactic	[201, 202]
TACI-Ig	Prophylactic	[203]

Nevertheless blockade of cytokines is more effective in the prevention of Th2 inflammation rather than the induction of Treg cells. Apart from the IL2: Anti-IL-2 none of the other studies targeting cytokines shows an effect on regulatory populations. In these cases it is important to take into account that the blocking antibody is present at the time of inflammation thus removing an important intervenient of the inflammatory process. IL-33 is not directly involved in the inflammatory process but rather on the very early process of sensitization[204, 205] orchestrating innate and adaptive immunity in a unique fashion via

basophils, mast cells, eosinophils, innate lymphoid cells, NK and NKT cells, nuocytes, Th2 lymphocytes and a $CD34^+$ precursor cell population[204]. It would be interesting to see if the anti-IL33 is able to induce a regulatory population, perhaps through a modulatory effect on the DCs. Some immune modulators like the anti-TIM-3 and anti-4-1BBL show a remarking increase in the levels of IFNγ [196, 199] suggesting a Th1 deviation [196] as the mechanism of regulation of a Th2 response.

Induction of transplantation tolerance with MAb.

Most animal studies have been performed in mice, although rats and nonhuman primates have been tolerized to foreign antigens and transplants with MAb. Transplant rejection or tolerance does not depend exclusively on the degree of mismatch between host and donor. Different strains of mice exhibit distinct behavior concerning the capacity to reject or become tolerant to transplants [206, 207], which may be further modulated upon exposure to infectious microorganisms. Active viral infection, with lymphocytic choriomeningitis virus (LCVM), at the time of tolerance induction with costimulation blockade and DST can prevent the effectiveness of the tolerization treatment leading to graft rejection [208-210]. However, this effect seems to be virus-specific, with some viral infections (e.g. murine cytomegalovirus and vaccinia virus) not leading to graft rejection [209]. A persistent viral infection with lymphocytic choriomeningitis virus clone 13 can also prevent the establishment of transplantation tolerance with a costimulation blockade-based regimen, and the antibody treatment can prevent an effective antiviral immune response [210]. Furthermore, the persistence of memory T cells elicited by

prior exposure to infectious agents has been reported as a barrier to the induction of transplantation tolerance, even in the absence of concomitant infection [211]. This phenomenon is generally known as heterologous immunity. An explanation for these findings may be the cross-reactivity between viral and transplantation antigens [209, 212]. However, these obstacles do not seem to be complete. With coreceptor blockade, instead of costimulation blockade, it has been possible to induce tolerance to allografts in mice or rats previously primed to donor antigens [155, 157]. In addition, infection of tolerized mice (with anti-CD4+ DST) with murine influenza PR8, at d 7 following heart transplantation, did not lead to impaired antiviral responses or to graft rejection [213].

Table 2. Therapeutic MAb Used to Induce Transplantation Tolerance

Antibody Target	Transplantation	Reference
CD2	Delayed rejection of islet allografts and xenografts	[214]
CD3	Tolerance to hearts allografts	[215]
CD3+CD2	Long term acceptance to heart grafts	[216]
CD4	Tolerance to antigens	[153, 217]
CD4+CD8	Tolerance to minor mismatched skin, MHC-mismatched and xenogeneic heart grafts	[153, 161]
CD4+CD8+CD154	Tolerance to MHC-mismatched skin	[141]
CTLA4-Ig	Xenogeneic pancreatic islets	[218]
CD45RB	Long term survival of MHC mismatched islets and kidney transplants	[219, 220]
CD45RB+CD154	Long term survival of MHC-mismatched skin	[221]
CD134(OX40L)	Long term survival of minor mismatched heart allografts	[222]
CD134L+CTLA4-Ig	Long term acceptance of MHC-mismatched hearts grafts	[223]
CD154	MHC-mismatched pancreatic islets	[224]
CD145+CD8dep	Tolerance to minor mismatched skin	[162, 164]
CD154+CTLA4-Ig	Long term acceptance of MHC mismatched skin	[225, 226]
LFA-1	Tolerance to soluble antigens	[152]
LFA1+ICAM-1	Long term acceptance of MHC-mismatched hearts grafts	[227]
IL-2+IL-15 Ig	Long term acceptance of MHC-mismatched islets in diabetic NOD mice	[228]

In general, the hierarchy of strains, from the easiest to tolerize to the most difficult, is as follows: C3H, CBA/Ca, DBA/2, BALB/c, and C57Bl/6. Apparently, these properties are not primarily due to the MHC molecules present in each strain. For instance, B10.BR mice, expressing identical MHC molecules to CBA/CA mice, are as difficult to tolerize as C57Bl/6 with whom they share only minor antigens[206] . The genetic basis of strain variability is still unknown.

Several studies have used T-cell receptor (TCR) transgenic animals, where all T cells share the same TCR. Even in these mice, it has been possible to use MAb, such as non-depleting CD4 or CD154, to induce a state of dominant tolerance where the de novo peripheral generation of Treg cells can be demonstrated [217]. It should be noted that prior to tolerization, these TCR-transgenic animals have no demonstrable CD4+CD25+Foxp3+ Treg cells.

The capacity to accept a transplant also depends on the graft itself. In general, the greater the genetic disparity between donor and recipient, the more difficult it becomes to prevent rejection. The donor strain itself may also contribute to different outcomes in terms of tolerance. For example, BALB/c grafts are harder to tolerize than C57Bl/6 grafts in CBA/Ca recipients [141, 229]. In addition, different organs have been shown to have distinct requirements for succumbing to tolerance induction [230, 231]. Vascularized grafts are, in general, more easily accepted than non-vascularized grafts, such as skin. Acceptance of the liver is relatively easy to achieve, with many different liver allografts being spontaneously accepted by permissive strains without any treatment [232]. Kidney allografts are also occasionally spontaneously accepted in rodents, although not as consistently as liver [231]. In contrast, pancreatic islets and heart allografts are usually rejected in the absence of therapeutic intervention. It should be

noted that in diabetic animals, such as non-obese diabetic (NOD) mice, it is more difficult to induce tolerance to islet allografts presumably because a primed autoimmune response must be overcome in addition to the allogeneic barrier [228, 233]. The rejection of small intestine seems to be more vigorous than the above mentioned organs [231, 234]. However, skin grafts are even more difficult to tolerize as some treatments, capable of preventing rejection of heart allografts, are ineffective in inducing long-term survival of skin allografts[235-238]. It is known that both CD4+ and CD8+ T cells can contribute to skin graft rejection, with each population capable of rejecting grafts independently [217].

Several antibodies and fusion proteins have been shown to be effective at inducing dominant transplantation tolerance, either on their own or in combination (see Table 2). It is interesting to note that virtually all MAb able to induce peripheral tolerance, target molecules involved in the formation of the immune synapse: the coreceptor molecules CD3, CD4, and CD8 [153, 216]; CD45RB [219]; the costimulatory molecules CD154 and CD28 [218, 224, 226]; and the adhesion molecules leukocyte function-associated antigen 1 and intercellular adhesion molecule-1 [152, 227]. It is thus possible that tolerance induction requires T-cell activation under suboptimal conditions, i.e., in the presence of reagents that interfere with efficient T-cell activation, although this does not extend to conventional immunosuppressive agents [139]. It has been reported that successful induction of dominant tolerance following coreceptor blockade leads to expansion of Treg cells [162], and elimination of alloreactive cells through activation-induced cell death [225, 239]. Some conventional immunosuppressive drugs, such as corticosteroids and calcineurin inhibitors like cyclosporine-A and tacrolimus, by preventing T-cell activation and activation-induced cell death can abrogate the induction of

dominant tolerance [225, 239-241]. The use of donor BM to induce tolerance by deletion of alloreactive clones does not seem to be affected by those drugs [211]. Other immunosuppressive drugs, such as rapamycin or mycophenolate mofetil, do not seem to prevent tolerance induction and may be useful in addition to the tolerogenic MAb [225, 241]. The impact of the novel immunosuppressant FTY720, which induces a reversible sequestration of T cells in secondary lymphoid organs, in tolerance induction has not been established [242]. Given that Treg cells can be found in tolerated allografts[159] , it will be important to assess whether the impact on T-cell migration may prevent the onset of dominant tolerance.

Induction of Tolerance With MAb in Autoimmunity

The use of MAb to restore the state of dominant regulation has been tested in several animal models of autoimmune and immune-inflammatory dis-ease. Some of the most common examples are listed in Table 3. Such animal models can be divided into three broad categories: (1) animals developing the dis-ease spontaneously, (2) animals where the disease is induced experimentally, and (3) TCR-transgenic animals where most or all the T cells are specific for a self-antigen. It should be noted that some animals, initially described as developing an autoimmune disease spontaneously, were later found to develop the disease following an environmental challenge. This is the case for SKG mice, carrying a mutation of the ZAP-70 gene, that develop chronic autoimmune arthritis with features closely resembling rheumatoid arthritis (RA) [7]. In spite of initial reports suggesting that autoimmune arthritis was spontaneous, it was recently shown that the disease is triggered by Dectin-1 agonists such as

zymosan [243]. It is also important to note that the immune systems of most animals used in animal models of autoimmune disease behave as lymphopenic [244]. Lymphopenia is a common feature in animal models of autoimmunity, appearing to be associated with the breakdown of tolerance [244]. In transplantation experiments it was shown that T-cell depletion in advance of treatment with costimulation blockade + DST constituted a barrier for tolerance induction [223]. Apparently, this effect is because of the acquisition of functional memory T cells [245]. It remains to be established whether tolerizing regimens, successful in inducing tolerance in pressensitized recipients [155, 157], will remain effective when used with lymphopenic animals.

Some of the most common animal models of autoimmune pathology are as follows:

Autoimmune diabetes: NOD mice spontaneously develop insulin-dependent diabetes and are a model of type I diabetes mellitus. Disease in these animals, as in humans, appears to be of autoimmune etiology that is heavily influenced by both genetics and environment [221, 246]. The similarity between diabetes in the NOD mice and humans is extensive, including the influence of sex on the incidence of disease: whereas about 80% of female mice at 6 months of age are diabetic, only 20% of males develop the disease [247]. BB rats also develop autoimmune diabetes spontaneously but, as in humans, the disease incidence is similar in both sexes and not affected by gonadectomy or androgen administration [248]. The immune system of these rats is severely lymphopenic. Another rat model of diabetes is the LEW.1WR1 rat ($RT1^{u/u/a}$) where the disease occurs spontaneously with a cumulative frequency of approximately 2% at a median age of 59 d. Both sexes are affected, and islets of acutely diabetic

rats are devoid of β cells whereas α and δ cell populations are spared [249].

Table 3. Animal Models of Autoimmune Diseases

	Associated human disease	Reference
Mouse models		
Spontaneous		
NOD	Type 1 diabetes	[221]
NZBXNZW	SLE	[250]
MRL/lpr	SLE	[251]
K/BXN	RA	[225, 252]
T/R^- (TCR transgenic to MBP)	MS	[253]
Induced		
DBA/1+Type II collagen in IFA	RA	[254-256]
SKG+Dectin-1 agonists	RA	[7, 243]
BALB/c + Spinal cord homogenate in CFA	MS	[257]
SJL + PLP in CFA	MS	[258]
SLJ + Thyroglobulin in CFA	Autoimmune Thyroiditis	[259]
Rat models		
Spontaneous		
BB/wor	Type 1 diabetes	[260]
Komeda diabetes-prone (KDP)	Type 1 diabetes	[261]
Lewis (LEW.1AR1/Ztm)	Type 1 diabetes	[262]
Induced		
Lewis + retinal pigment epithelium-specific 65-kDa protein peptides	Autoimmune uveitis	[263]
Lewis + MBP in CFA	MS	[264]
Wistar + MBP in CFA	MS	[231]
Wistar + testis homogenate	Autoimmune orchitis	[265]

CFA, Complete Freund's Adjuvant; IBD, Inflammatory Bowel Disease; IFA, Incomplete Freund's Adjuvant; MBP, Myelin Basic Protein; MS, Multiple Sclerosis; RA, Rheumatoid Arthritis; SLE, Systemic Lupus Erythematosus

RA: the most commonly used animal models of autoimmune arthritis are mice and rats in which the disease is induced with type II collagen and incomplete Freund's adjuvant [254-256]. In these cases, the disease usually affects larger joints of the limbs and is self-limited. K/BxN mice spontaneously develop a progressive joint-specific autoimmune disease between 3 and 5 wk of age. Pathology of disease in K/BxN mice is

similar to human RA, with pannus formation, synovial hyperplasia, increased synovial fluid volume, and chaotic remodeling of cartilage and bone in the distal joints in the later stages [225, 252]. SKG mice, harboring T cells more resistant to TCR stimulation, also develop chronic autoimmune arthritis. Although disease in K/BxN mice is mediated by antibodies and can be adoptively transferred to healthy recipients by transferring the serum, in SKG mice arthritogenic T cells are sufficient to cause the disease as their adoptive transfer into lymphocyte-deficient hosts triggers autoimmune arthritis [7].

Multiple sclerosis: EAE is characterized by T cell-mediated destruction of the myelin sheath in the central nervous system. Myelin basic protein, myelin/oligo-dendrocyte glycoprotein, and proteolipid apoproteins when administered with adjuvant to mice or rats can induce autoreactive T cells that cause the disease [258, 266, 267]. The same outcome has been reported following the administration of syngeneic mouse spinal cord homogenate in complete Freund's adjuvant [257]. TCR transgenic mice in the RAG/- background, in which all T cells express a TCR directed to a myelin protein, spontaneously develop EAE with 100% incidence at 8 months [253, 268]. Interestingly, the disease is abrogated by the presence of additional CD4+ T cells from wild-type syngeneic mice [269, 270].

Systemic lupus erythematosus (SLE): MRL/lpr mice spontaneously develop a disease similar to SLE, because of defective apoptosis of activated B cells as the mice are deficient in Fas/FasL [250]. Other mouse strains, such as the NZB x NZW and the BXSB, also develop a SLE-like syndrome that appears to be influenced by multiple genes [250].

Several MAb have been tested for the treatment of autoimmune dis-eases. In many cases such MAb do not aim to restore tolerance but

rather to control the inflammatory process (such as the anti-tumor necrosis factor a or the anti-interleukin-1 MAb)[271], or induce immunosuppression by elimination of pathogenic lymphocyte populations (such as T-cell depletion with CAMPATH or B-cell depletion with anti-CD20) [272, 273]. However, tolerogenic MAb have also been reported to have a beneficial effect on the prevention or treatment of autoimmune pathology, although in many cases the true re-establishment of dominant self-tolerance based on T-cell regulation remains to be confirmed (see Table 4).

CD3: therapy with nonmitogenic CD3 MAb has been shown to prevent and revert type 1 diabetes in NOD mice and in virus-induced autoimmune diabetes [274-276]. Different from most other tolerogenic MAb, anti-CD3 seems to be more effective if used after the onset of disease, apparently because activated T cells are the main targets of this treatment [275]. In autoimmune arthritis the use of this anti-body was not shown to be as effective as in type 1 diabetes, but in both systems, treatment causes a reduction in serum interferon-γ and an increase in interleukin-4, interpreted as deviation to a Th2 response [277]. Anti-CD3 MAb were also shown to lead to an increase in Treg cells able to suppress diabetogenic T cells and maintain a state of dominant tolerance [167, 278].

CD4: following initial reports showing a beneficial effect of anti-CD4 on the treatment of EAE in rats [149], it has been shown prevent the onset of diabetes in NOD mice, as well as preventing pancreatic islet damage in recently established disease, with the animals tolerating islet trans-plants from prediabetic syngeneic donors or even islet allografts [208, 279-282]. The same MAb have also been shown to prevent the onset of experimental autoimmune arthritis as well as to ameliorate overt arthritis [283-285].

Several other MAb, such as the ones targeting CD154, CTLA4-Ig, and CD134L, have been shown effective in preventing or treating several experimental autoimmune diseases (see Table 4).

Table 4. Targets of Therapeutic MAb in Experimental Autoimmune Diseases

MAb	Animal/disease	Outcome	Reference
CD2	Lewis rats (EAM)	Prevents onset	[286]
	Lewis rats (EAE)	Prevents onset	[287]
	BB/wor rats (type 1 diabetes)	Prevents onset	[260]
CD3	NOD mouse (type 1 diabetes)	Ameliorates established disease	[167, 274-276]
	DBA/1 mouse (CIA)	Delayed onset with reduced severity	[277]
CD4	NOD mouse (type 1 diabetes)	Prevents onset	[208, 279-281]
	NZB/NZW mouse (murine SLE)	Ameliorates established disease	[288]
	DBA/1 mouse (CIA)	Prevents onset and ameliorates established disease	[289, 290]
CD30L	NOD mouse (type 1 diabetes)	Prevents onset	[291]
OX40L	SJL mouse (EAE)	Ameliorates established disease	[258]
	C.B-17 SCID (IBD)	Ameliorates established disease	[292]
CTLA4-Ig	BALB/c mouse (EAE)	Ameliorates established disease	[257]
	NZB/NZW mouse (murine SLE)	Prevents onset	[293-296]
	BXSB mouse (SLE)	Delayed onset with reduced severity	[297]
CD154	Marmoset monkey (EAE)	Prevents onset	[298]

	B6/A (C57BL/6XA/J) (AOD)	Prevents onset	[299]
	B10.BR mouse (EAU)	Ameliorates established disease	[300]
	SJL mouse (EAT)	Less severity	[259]
	NZB/NZW mouse (murine SLE)	Prevents onset	[292, 301-303]
CD40	Marmoset monkey (EAE)	Prevents onset	[304]
CD137	DBA/1 mouse (CIA)	Prevents onset	[305]
	NZB/NZW mouse (murine SLE)	Ameliorates established disease	[306]
	C.B-17 SCID (IBD)	Ameliorates established disease	[307, 308]
	C57BL/6 (EAE)	Ameliorates established disease	[267]
LFA-1	NOD mouse (type 1 diabetes)	Prevents onset	[309]

EAE, Experimental Allergic Encephalomyelitis; EAM, Experimental Autoimmune Myelitis; AOD, Autoimmune Ovary Disease; CIA, Collagen Induced Arthritis; EAU, Experimental Autoimmune Uveoretinitis; IBD, Inflammatory Bowel Disease; MS, Multiple Sclerosis; SLE, Systemic Lupus Erythematosus.

MAb have been used either to prevent the onset of experimental autoimmune diseases, or to treat overt autoimmunity. It is usually assumed that newly formed T cells exported from the thymus create an obstacle for dominant tolerance maintenance. In some stringent experimental systems, the use of thymectomized animals can facilitate maintenance of the tolerant state [221]. Dominant tolerance, however, has been induced in either euthymic or thymectomized animals in many experimental systems, including stringent MHC-mismatched skin transplantation [141]. The presence of the thymus is required for the induction of tolerance based on mixed-chimerism, where thymic deletion of alloreactive clones occurs [310]. For the prevention of autoimmunity, animals are often treated from the neonatal period [299]. In some experiments, anti-CD3 MAb have been shown to be successful in treating

overt diabetes in NOD mice, restoring a state of dominant tolerance mediated by Treg cells [274, 275]:

Tolerance vs. Immunosuppression

Long-term allograft survival following MAb treatment does not prove dominant tolerance has been successfully induced. To demonstrate tolerance, it is necessary to confirm that the immune system is fully competent to mount a response against antigens other than the tolerated ones. Usually, a simultaneous transplant of tolerated-type and unrelated third-party grafts is used, to confirm that tolerant animals remain able to reject specifically the third-party transplant (see Fig. 4).

The Antigen Specificity of the Tolerant State

The putative antigen-specificity of the tolerant state is frequently demonstrated simply by grafting a third-party and a tolerated type transplant, and observing that only third-party grafts are rejected. However, given the possible differences in the "rejectability" of the different types of grafts (see Subheading 2.1.), such results should be approached with caution [38]. Ideally, these studies should be performed in crisscross experiments: mice should be tolerized, in independent experiments, to transplants of different types. Then both groups should be tested for capacity to reject the appropriate third-party grafts (see Fig. 4).

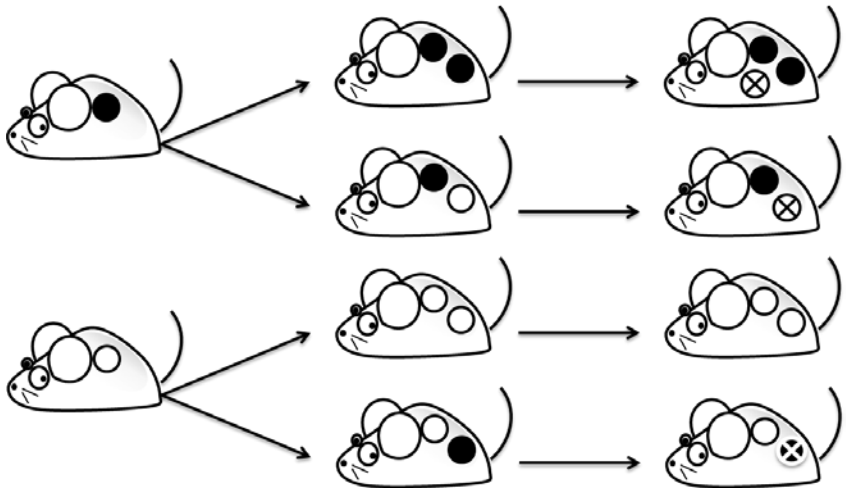


Figure 4. Crisscross experiment to demonstrate antigen specific tolerance. To account for possible strain variations in tissue "rejectability", tolerance can be assessed in mice tolerized to two different strains. The antigen specificity of the tolerant state will determine that animals from both groups reject grafts from the third-party strain. The same sort of experiment can be used to test the antigen specificity of putative regulatory T cell populations following their adoptive transfer into lymphocyte-defined animals.

Alternatively linked suppression can be used as an exquisite and straightforward assay for both specificity and the dominant nature of tolerance (see below).

Dominant Tolerance

A direct way to assess the dominant tolerance status of an animal is through the infusion of T cells with the capacity to reject the graft. In such a challenge, T cells obtained from the spleens of naïve animals can be used [153, 160]. If the transfused cells are allowed to coexist with the tolerant ones for 6 wk, it becomes possible to demonstrate that they themselves have been rendered tolerant by ablating the initial population of T cells with depleting MAb directed to a specific marker [142, 162]. This

recruitment of T cells toward a regulatory function is generally known as infectious tolerance.

Instead of using T cells from naïve wild-type animals, it is possible to transfuse TCR-transgenic T-cells specific for the tolerized alloantigens [267]. This experimental setting allows the study of dominant tolerance effects on the cells being subjected to active regulation [267].

An alternative strategy to demonstrate dominant tolerance is to deplete putative populations of Treg cells, such as CD4+ or CD25+ T cells, unleashing aggressive cells to mediate rejection. It should be noted, however, that tolerizing treatments are often associated with a partial deletion of the alloreactive clones [225, 239]. In addition, besides the major populations of Treg cells (identified as CD4+CD25+ and CD4+CD25) other minor populations of Treg cells may persist. This seems to be the case for CD8+ or double negative (CD4 CD8) Treg cells [231, 311] in the light of which, it is not surprising that, in some cases, ablation of the major Treg cell populations—such as the CD4+ or the CD25+ cells—does not lead to the rejection of tolerized allografts.

Linked suppression has been used as an efficient assay for confirmation of both antigen-specificity of tolerized animals and for the dominant state of tolerance [141, 312]. These experiments are based on the observation that, once dominant tolerance has been established to a set of antigens present in an allograft, new transplants with cells simultaneously expressing tolerated antigens and third-party antigens are not rejected (see Fig. 5) [163]. These data have been interpreted as evidence that Treg cells specific for the tolerated antigens actively suppress the aggressive cells directed to the “linked” third-party antigens. With time, those aggressive clones will themselves become tolerized, because animals start to accept transplants with the third-party antigens in

the absence of the tolerated ones. It should be noted that third-party grafts, where the tolerated set of antigens is absent, are readily rejected in control animals. Linked suppression has been demonstrated in situations where tolerance is induced with coreceptor blockade, costimulation blockade or a combination of the two [141, 163, 164].

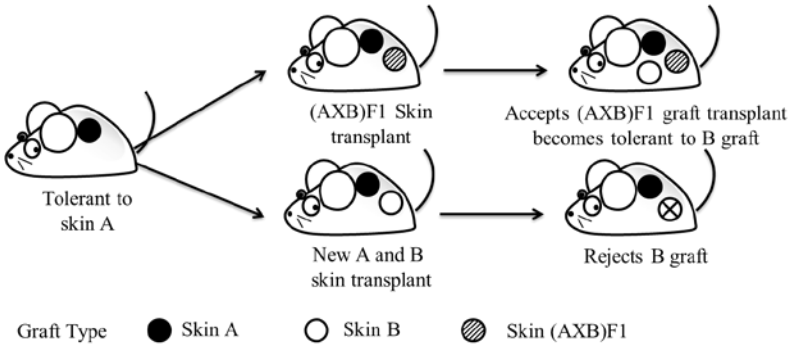


Figure 5. Linked suppression. Animals tolerized to skin grafts of type A accept skin grafts from (AXB) F1 donors, where third part B antigens are endogenous to cells that also express A antigens (top row). However, B type grafts are readily rejected, even if a concomitant tolerated A type graft is given (bottom row). Furthermore, the animals that accept the (AXB) F1 grafts with the "linked" antigens become tolerant to grafts of the third party B type.

Although MAb have been successful in inducing dominant tolerance in animal models of allergy, transplantation and autoimmune diseases, it will be important to investigate whether similar strategies can in different contexts give rise to different outcomes in specific regulatory populations. These experimental systems have been useful in understanding the biology of Treg cells and the suppressive mechanisms maintaining dominant tolerance. But besides such important contributions toward the basic understanding of the immune system and its regulation, there is a potential for clinical application of tolerogenic MAb. Besides the above immune disorders the use of MAb as immune modulators is now

being adopted, in the context of inhibitor generation in protein substitution therapies. Recent observations have shown that MAb equivalent to the ones used in murine studies can be successfully used to induce tolerance in primates [217, 240, 313]. Furthermore, a recent clinical trial has shown a beneficial effect of tolerogenic CD3 MAb in autoimmune diabetes [314]. There are, therefore, reasons for optimism concerning the clinical use of tolerogenic antibodies in human immune pathology.

Aims of the Thesis

Over the years several strategies have been developed to induce immune tolerance in a range of immune pathologies. One reagent that has proven effective in inducing long-term tolerance in transplantation and autoimmunity is a non-depleting isotype of anti-CD4 MAb. However, the induction of immune tolerance with anti-CD4 has been shown easier to achieve to alloantigens in transplants than to single proteins, such as FVIII in mouse models of hemophilia. These observations can be considered surprising given the greater "mismatch" in models of transplantation compared to hemophilia.

The current thesis addresses the issue of tolerance induction to proteins, such as allergens or FVIII. We assessed how adjuvants can play a role in facilitating tolerance induction and, finally, what are the different mechanisms leading to tolerance to the same foreign antigens when they are delivered as part of a transplanted tissue or as a protein in alum.

As a consequence, the first chapter of the results describes a variety of experiments that were designed to demonstrate whether it is possible to induce tolerance to allergens (house dust mite) or a model antigen (OVA) in a mouse model of allergic asthma. We found that CD4 blockade is remarkably effective in inducing long term tolerance to those antigens, while the immune system remains fully competent to respond to unrelated antigens and inclusively to develop allergic airways disease to those unrelated antigens.

The second chapter of the results assessed whether an adjuvant (alum) can be used to facilitate the induction of immune tolerance to FVIII in a mouse model of hemophilia. We found that indeed alum can facilitate tolerance, even in mice that have been primed in advance of tolerance induction.

Finally, the third chapter of the results describes experiments where we investigated the mechanisms underlying the induction of tolerance to foreign proteins in alum, and how those mechanisms differ from tolerance induced to the same antigens when they are presented within transplanted tissue. We found that tolerance to proteins in alum is mediated by recessive mechanisms based on the apoptosis and anergy of effector cells, together with dominant mechanisms that are Foxp3-independent and IL-10-dependent.

The use of monoclonal antibodies to neutralize key molecules involved in the differentiation of inflammatory cells and to neutralize cytokines and other molecules involved in the inflammatory process is becoming exceptionally popular. There is a wealth of such components in clinical trials and some already in clinical use. Although promising anti-CD4 hasn't yet been fully availed by the scientific and clinical community. Targeting a key molecule in the activation of the helper T cell this tolerogenic agent can influence the effector and regulator populations tipping the balance of the immune system towards a specific, robust tolerance.

It is the purpose of this work to demonstrate the effectiveness of anti-CD4 in inducting tolerance in Th2 biased immune disorders while sparing the system to respond to unrelated antigens. We also aim to show

that primed cells are still susceptible to the tolerizing effect of CD4 blockade. In transplantation and autoimmunity CD4 is described to induce putative Tregs nevertheless other mechanisms have come forward and we hope to demonstrate that in soluble protein tolerance induction the mechanism has a recessive and dominant component independent of $Foxp3^+$ Tregs. We also hope to understand if adjuvants can have a beneficial role in potentiating the tolerogenic effect of the anti-CD4 MAb.

Lastly and taking together the objectives stated above it seems pressing to evaluate if inducing tolerance in a $Foxp3^+$ Treg independent fashion can be combined with the $Foxp3^+$ Treg dependent mechanism to boost tolerance induction.

Results

Prevention of house dust mite induced allergic airways disease in mice through immune tolerance

Introduction

The control of deleterious immune responses causing diseases, such as allergy, autoimmunity and transplant rejection, has been one of the main objectives of immunologists. Moreover, the global prevalence of this type of diseases has been steadily increasing.

Several strategies have been recently described to induce tolerance to allergens thus preventing allergic airways disease [81, 315-317]. In brief, they can rely on the induction of dendritic cell (DC) populations or regulatory T cells (Treg) able to control pathologic T cell clones, in a process where IL-10 and TGF-β can participate [47, 121, 315, 318-321]**.** In addition, disease prevention may be achieved by skewing the immune response from a Th2 to a Th1 phenotype [196].

In fact, the realization of the critical importance of T cells in the pathogenesis of allergic airways disease was well demonstrated by studies where anti-CD4 monoclonal antibodies (MAbs) causing the depletion of this T cell subset could prevent the disease in mice [322]. Such pre-clinical studies with CD4 T cell depletion provided the rationale for clinical trials with depleting anti-CD4 MAbs where the short-term benefit observed was probably associated with transient immune suppression [323]. As a

consequence, the interest has shifted towards MAbs capable of blocking molecular interactions but without leading to direct cell lysis.

Some reports have shown prevention of allergic airways disease following the blockade of T cell co-stimulatory or co-receptor molecules with non-depleting MAbs, but it remains unclear whether long-term antigen-specific tolerance is achieved or what are the mechanisms involved [178, 179, 324, 325]. We now describe CD4 blockade at the time of exposure with a model antigen, ovalbumin (OVA), or a clinically relevant allergen, house dust mite (HDM), can induce antigen-specific tolerance and protection from allergic airways disease. The mechanism leading to antigen-specific tolerance without affecting protective immune responses (including Th2-type responses) to additional antigens is independent of a switch between a Th2-type and Th1-type immune response. Since CD4 blockade is achieved with a non-depleting MAb, T cells not activated by the antigen remain unaffected to mount protective immune responses towards unrelated antigens at a later time.

Tolerance induction by CD4 blockade is robust enough to be effective in pre-sensitized animals and even in animals where AHR was previously established. The tolerant mice show protection from allergic manifestations elicited by intranasal exposure to the antigen: they do not develop airways eosinophilia, goblet cell hyperplasia, production of Th2 cytokines in the lung, production of antigen-specific IgE or IgG1, and, importantly, do not develop airway hyperreactivity (AHR) in response to inhaled methacholine (MCh).

Materials and Methods

Experimental animals

BALB/c mice were bred and maintained under specific pathogen-free facilities. Animals were sensitized, at the times described in the text, by i.p. injection of 20 μg in 2.0 mg of endotoxin-free aluminum hydroxide (Alugel-S, Serva, Heidelberg, Germany) of OVA or β-LG (Sigma, St Louis, USA) previously run through a DetoxyGel column (Pierce, Rockford, USA), or HDM extract (Greer, Lenoir, USA). In all experiments animals were age and sex matched.

Ethics Statement

All experiments involving animals were approved by Direcção Geral Veterinaria (approval 018831). Mice were bred and maintained under specific pathogen free (SPF) conditions.

Antibodies and reagents

Non-depleting anti-CD4 (YTS177) [153] and the isotype control (YKIX302) MAbs were produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland), and purified from culture supernatants by 50% ammonium sulfate precipitation, dialyzed against PBS, and the purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Bronchoalveolar Lavage (BAL)

Airways were washed through the trachea with 3 ml of cold PBS 1% BSA (Sigma). The BAL was centrifuged, ressuspended in PBS, and the cells

counted with a hemocytometer. Differential cell counts were performed on cytospin samples stained with Giemsa-Wright (Sigma). At least 200 cells from each sample were counted, using blinded slides, to determine the relative frequency of each cell type. In addition, in some experiments eosinophilia was independently confirmed by flow cytometry using GR-1 (eBiosciences, San Diego, CA, USA), CCR3 (BD Pharmingen, San Diego, USA), and MHC-class II MAbs (produced in-house), with eosinophils identified based on the SSC/FSC profile and as the $GR1^{int}$MHCclass $II^{-}CCR3^{+}$ cells [326].

Quantification of immunoglobulins and cytokines

Serum titers of OVA-specific IgG1, IgG2a, and IgE were measured by ELISA using the following: IgG1 and IgG2a (SouthernBiotech, Birmingham, USA) with anti-OVA IgG1 standard from Serotec, Oxford, UK; IgE (BD-Pharmingen) with anti-OVA IgE standard from Abcam (Cambridge, UK). Cytokine titers were determined in fresh BAL and lung homogenates. Cytokine ELISAs were performed using the following kits: IL-4, IL-5 (BD-Pharmingen).

Histology

Lungs were perfused with 4% formalin solution (Sigma), collected and sectioned. Staining was performed using hematoxilin/eosin, and periodic acid-Schiff (PAS) stain. Photographs were taken using a Leica DM2500 microscope and a Leica DFC420 camera.

Respiratory mechanics and methacholine responsiveness

Airway responsiveness was determined 24 hours after last intranasal OVA challenge. Changes in the respiratory input impedance (Zrs) were

measured using a modification of the low frequency forced-oscillation technique (LFOT) in mice anesthetized with 10 µl/g of xylazine (2 mg/ml, Ronpum, Bayer, Germany) and ketamine (40 mg/ml, Merial, Lyon, France), tracheotomized and ventilated (FlexiVent, SciReq, Montreal, Canada). Mice were hyperventilated at 450 breaths/min and Zrs was measured during periods of apnea using a 16 s signal containing 19 prime sinusoidal frequencies as described elsewhere [327]. Calculation of airway resistance (R_{aw}), tissue damping (G) and tissue elastance (H) is obtained from the Zrs spectrum using FlexiVent software (SciReq). AHR was measured by exposure to an aerosol containing increasing doses of MCh (Sigma), following a baseline measurement after the delivery of a saline aerosol.

Statistical analysis

Statistical significance was determined using the two-tailed non-parametric Mann-Whitney test and *P* values <0.05 were deemed significant (*, *P*<0.05; **, *P*<0.01; ***, *P*<0.001).

Results

Co-receptor blockade with non-depleting anti-CD4 MAb prevents allergic sensitization in mice

Using a well established murine model of allergic airways disease we sought to determine if non-depleting MAbs targeting the T cell co-receptor

molecule CD4 were effective in preventing allergic sensitization with HDM or a model antigen (OVA).

BALB/c mice were sensitized with two i.p. injections of OVA-alum or HDM-alum on days 1 and 14, and challenged with 50 µg OVA or HDM i.n. on days 20, 21 and 22 (Figure 1A). Experimental groups were treated with 1 mg i.p. of anti-CD4 or an isotype control on the days before and after each immunization, and sacrificed 24 hours following the last intranasal challenge.

Mice treated with anti-CD4 had a marked reduction in BAL eosinophils when compared with sensitized animals, to levels similar to naïve animals or animals sensitized in the absence of the antigen (Figure 1B, and Figure S1). The absence of goblet cell hyperplasia and inflammatory infiltrate in the airways of anti-CD4 treated mice was confirmed by histology (Figure 1C). Furthermore, anti-CD4 treatment prevented effective generation of Th2-driven OVA- and HDM-specific IgG1 and IgE (Figure 1D). We could not detect Th1-driven antigen-specific IgG2a in any animal (not shown). Animals treated with anti-CD4 showed a marked reduction of IL-4 and IL-5 in lung homogenates to levels similar to naive animals (Figure 1E). Importantly, we found no evidence for Th1 or Th17 deviation (as inferred by levels of IFNγ or IL-17), nor increased levels of the immune-regulatory cytokine IL-10 (not shown). Cytokines in BAL were similar to lung homogenates (not shown).

To study the functional impact of the treatment we assessed AHR in response to increasing doses of inhaled MCh. Our data show that anti-CD4 treatment prevented AHR (Figure 1F and Figure S2).

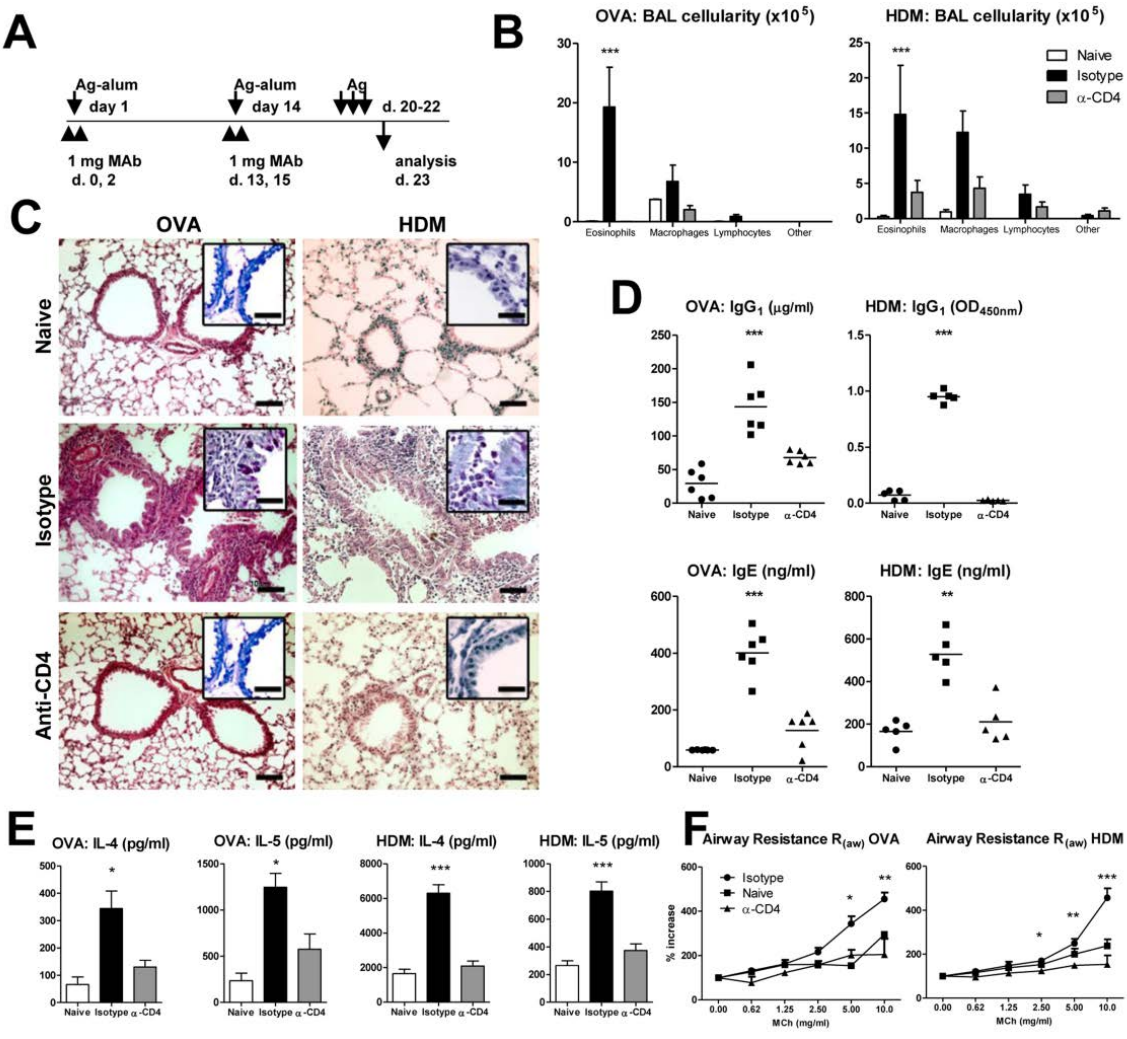


Figure 1. Prevention of allergic sensitization with anti-CD4 MAb. (A) Female BALB/c mice were sensitized with 20 μg OVA-alum or 50 μg HDM-Alum i.p. and challenged with 50 μg OVA or HDM in saline i.n. on the indicated days. Some animals were treated with 1 mg anti-CD4 or an isotype control i.p. as shown. Naive mice, not subjected to any intervention, were also studied as a control group. (B) Cellular composition of the BAL. Animals treated with anti-CD4 have less eosinophils in the BAL (n=6, *** P<0.001). (C) Histological sections of lung tissue were stained with haematoxylin/eosin and PAS (inset). Anti-CD4-treated mice have reduced inflammatory infiltrate and goblet cell hyperplasia, to levels similar to naive controls. Bars represent 10 μm (2.5 μm in the inset). (D) Quantification of serum OVA- and HDM-specific IgG1 and IgE. Anti-CD4 MAb treated mice show a significant reduction of the Th2-driven immunoglobulins (n=6, *** P<0.001 and ** P<0.01 as indicated). (E) The Th2 cytokines IL-4 and IL-5 were down to basal levels

in lung homogenates of anti-CD4 treated mice (n=6, * P<0.05 and *** P<0.001). (F) Invasive measurement of respiratory mechanics shows that animals treated with anti-CD4 MAbs had reduced airway resistance to increasing doses of inhaled MCh, when compared with sensitized control animals (n=8). Data (B-F) are representative of at least three independent experiments.

The tolerance state is maintained following clearance of the MAb

We then studied whether tolerance induction with non-depleting anti-CD4 would protect the animals from subsequent exposure to the same antigens, at a time the therapeutic MAb had been cleared. For this purpose treated mice with anti-CD4 at the time of initial sensitization with OVA or HDM, and the same mice were again immunized with the same antigens 50 days following the initial treatment (Figure 2A).

Sensitization with OVA-alum or HDM-alum did not lead to airways eosinophilia in mice previously exposed to the same antigens under the cover of non-depleting anti-CD4 (Figure 2B and Figure S1). Furthermore, the treated mice also failed to produce antigen-specific IgG1 and IgE to OVA-alum and HDM-alum (Figure 2C). And importantly, AHR to increased concentrations of inhaled MCh was also absent in anti-CD4 treated mice (Figure 2D and Figure S2).

We could therefore conclude that a short course of anti-CD4 was leading to long-term effects. Although the MAb we used (clone YTS177) is known to have a non-depleting isotype [153], and we confirmed anti-CD4 treatment was not directly leading to T cell lysis (not shown), we had to confirm the treated mice remained immune competent.

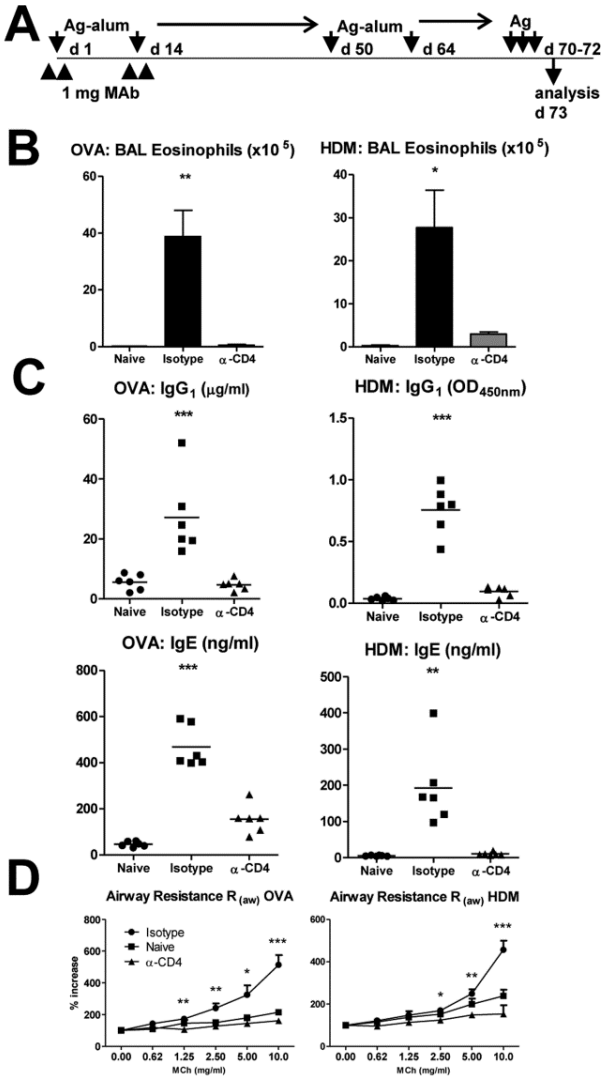


Figure 2. Tolerized mice resist subsequent sensitization and challenge. (A) BALB/c mice were initially sensitized with OVA or HDM under the cover of anti-CD4 as described in Figure 1. Those mice were sensitized with the same antigens on days 50 and 64, and subsequently challenged i.n. (B) Animals treated with anti-CD4 were protected from BAL eosinophilia (n=6, ** P<0.01 for OVA; * P<0.05 for HDM). (C) CD4-blockade prevented production of IgG1 and IgE in subsequent sensitizations (n=6, *** P<0.001 and ** P<0.01 as indicated). (D) Tolerance to OVA or HDM prevented AHR to inhaled MCh (n=6). Data are representative of three independent experiments

Tolerant mice remain immunocompetent

In order to study the antigen-specificity of tolerance induction, we used a second unrelated antigen: β-lactoglobulin (β-LG). We compared immune responses to OVA and β-LG, since these are two defined antigens with similar characteristics, while HDM is a complex protein extract containing many distinct antigens.

BALB/c mice were treated with non-depleting anti-CD4 MAb together with OVA-alum (tOVA) or β-LG-alum (tβ-LG) in order to establish immune tolerance to those antigens (Figure 3A). At day 50 the animals were immunized with the same antigen used at the time of tolerization (day 0) or with the second unrelated antigen. All mice were subsequently challenged i.n. with the same antigen used at day 50. All animals remained protected from mounting airways inflammation in response to the antigen used for tolerization, but fully competent to undergo a Th2 response to the second antigen leading to airways eosinophilia, production of IgE and IgG1, and AHR (Figure 3B-D and Figure S2). These results suggest that CD4-blockade affects specifically the T cells that are being activated at the time of treatment, and sparing non-activated cells, thus leading to antigen specific tolerance where immune responses against different antigens are not suppressed.

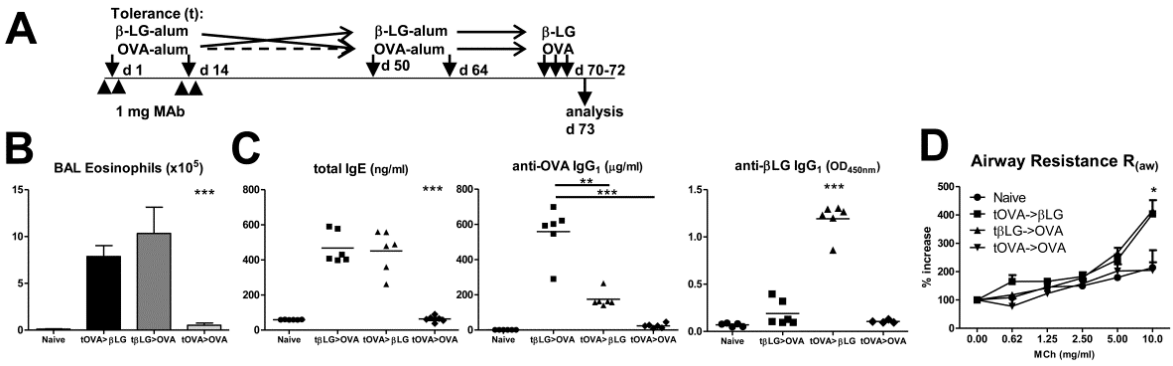


Figure 3. Mice treated with anti-CD4 are competent to respond to unrelated antigens. (A) Mice initially tolerized to OVA or β-LG (as described in previous figures) were sensitized i.p. with a different antigen at days 50 and 64, and challenged i.n. with the same antigen used at day 50. (B) Only animals tolerized to the same antigen used for sensitization at day 50 were protected from BAL eosinophilia (n=6, *** P<0.001). (C) Tolerance induction to OVA did not prevent subsequent production of β-LG-driven IgE or IgG1, conversely, tolerance to β-LG did not hamper the generation of OVA-specific IgG1 or IgE (n=6, *** P<0.001). (D) AHR in response to MCh was observed in animals tolerized to an antigen different from the one used for subsequent sensitization (tOVA >β-LG and tβ-LG >OVA) (n=8, * P<0.05 at 10 mg/ml MCh). Data are representative of two independent experiments.

Tolerance can be achieved in sensitized mice.

To assess whether tolerance can be induced in pre-sensitized mice, BALB/c mice sensitized with OVA or HDM were treated with the same antigen under the cover of anti-CD4 50 days following initial sensitization (Figure 4A). For consistency with previous experiments we maintained the tolerance-inducing regime as two administrations of antigen-alum + anti-CD4 two weeks apart.

We found, both OVA- and HDM-sensitized mice treated with anti-CD4 were prevented from AHR, maintaining normal airway response to

increased concentrations of inhaled MCh (Figure 4B and Figure S2). We confirmed the efficient sensitization of all groups of immunized animals by the presence of antigen-specific IgG1 and IgE antibodies in sera (not shown), although the mice had not been exposed to prior airway inflammation. As a consequence, the protection from AHR is effective in spite of high titers of antigen-specific immunoglobulins – possibly representing an impact on the late-phase response, and dissociation between high IgE and AHR.

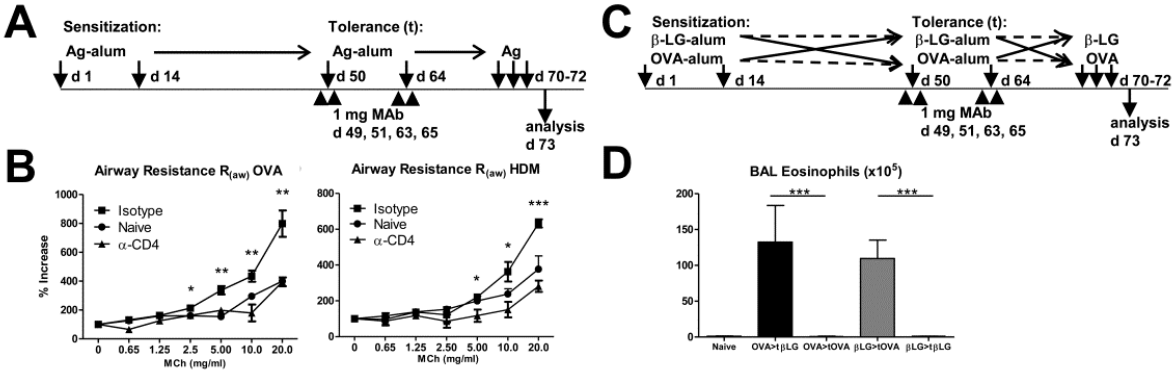


Figure 4. Tolerogenic effect of anti-CD4 treatment is antigen-specific and effective in sensitized animals. (A) BALB/c mice sensitized with OVA-alum or HDM-alum were tolerized to the same antigens on days 50 and 64, and challenged i.n. with the same antigens. (B) Sensitized mice subsequently treated with OVA or HDM under the cover of anti-CD4 showed protection from AHR (n=8 for OVA, n=6 to HDM). Data are representative of two independent experiments. (C) Mice were initially sensitized with OVA-alum or β-LG-alum, and tolerized to the same or a different antigen on days 50 and 64. All mice were challenged i.n. with the same antigen used for initial sensitization. (D) Mice treated with a different antigen together with anti-CD4 did not show reduced BAL eosinophilia (OVA >tβ-LG and β-LG >tOVA) while treatment with anti-CD4 and the same antigen used for sensitization showed a significant reduction of BAL eosinophilia (OVA>tOVA and β-LG >tβ-LG; n=6, *** P<0.001). Data are representative of two independent experiments.

We then assessed whether tolerance induction in pre-sensitized mice remained antigen-specific. BALB/c mice sensitized with OVA-alum or β-LG-

alum were treated with anti-CD4 MAb, 50 days following the initial intervention, in the presence of either the initial (OVA or β-LG) or a different antigen (β-LG or OVA, respectively; Figure 4C). Sensitized mice were protected from airway eosinophilia when treated with anti-CD4 in the presence of the same antigen used for sensitization (OVA >tOVA and β-LG >tβ-LG, Figure 4D). The protective effect was, therefore, not due to the persistence of the therapeutic antibody in circulation at the time of intranasal exposure to the antigen since mice immunized with a different antigens not present during anti-CD4 treatment (and therefore with equivalent doses of circulating anti-CD4 at the time of challenge) were not protected (OVA >tβ-LG and β-LG >tOVA). The observation that animals receiving the antibody treatment together with a different antigen than used for immunization develop inflammatory changes similar to untreated control animals, or animals exposed to alum in the absence of the antigen (Figure S1), are consistent with the antigen-specificity of the tolerance state described above.

Mice exposed to allergic airways disease can be protected from AHR following anti-CD4 treatment.

However, it is understood that the onset of inflammation in the airways becomes a significant hurdle for immune modulation leading to tolerance. Therefore, we investigated whether mice sensitized to OVA or HDM and exposed to the antigen i.n. could benefit from subsequent anti-CD4 treatment (Figure 5A). A single i.n. challenge with antigen in sensitized mice was sufficient to induce AHR (Figure S3).

We found that administration of HDM under the cover of non-depleting anti-CD4 30 days following induction of allergic airways disease was effective in preventing AHR following subsequent challenge with the same antigen (Figure 5B). We repeated the same studies with OVA with similar results (Figure 5B and Figure S2).

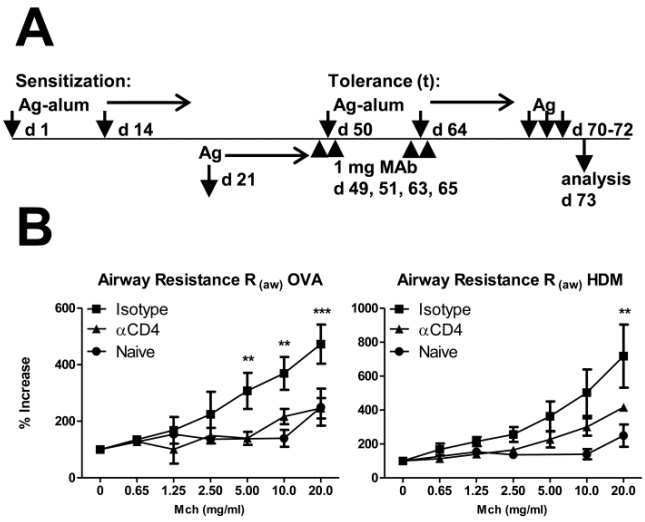


Figure 5. Protection from AHR in animals previously exposed to airways inflammation. (A) Balb/c mice sensitized and challenged i.n. with HDM-alum or OVA-alum were tolerized to the same antigens on days 50 and 64, and challenged i.n. with the same antigens. (B) The animals treated with HDM or OVA under the cover of anti-CD4 were protected from AHR in response to inhaled MCh (n=6, ** P<0.01 or *** P<0.001 as indicated). Data are representative of two independent experiments

Discussion

Our data shows that CD4 blockade is effective in inducing antigen-specific tolerance to a clinically relevant allergen (HDM), thus preventing the manifestations of allergic airways disease following intranasal allergen challenge: Th2 and eosinophilic infiltrate of the airways, goblet cell hyperplasia, and AHR. The tolerogenic treatment is not only effective in preventing the disease in naive animals, but also confers considerable protection to mice previously sensitized with the allergen. Although in different experiments we tested mice with different ages, we could not find an age-related difference in their response to induction of allergic airways disease (Figure S4).

It should be noted that the use of tolerogenic MAbs in transplantation, with the same objective of preventing an inflammatory response to non-self antigens, have resulted in a different outcome from what we have observed. In transplantation, tolerance induction in sensitized animals has been difficult to achieve, except when both anti-CD4 and anti-CD8 MAbs are combined [155], with anti-CD8 MAbs probably required to control pre-committed effector T cells. Co-stimulation blockade with anti-CD40L has also been reported as less effective than anti-CD4, requiring depletion of $CD8^+$ T cells even in non-sensitized animals [164, 165]. The obstacle created by sensitization in relation to tolerance induction is also evidenced by reports showing that heterologous immunity reduces the effectiveness of tolerogenic protocols in transplantation [211]. Our data shows that the anti-CD4 antibody treatment can also be beneficial to already sensitized animals. This may be due to the fact that the T effector cell frequency is significantly lower for allergens than for alloantigens and that the response

to allergens is predominantly restricted to the $CD4^+$ compartment. It is likely that such different outcome observed in transplantation versus allergy may be due to the induction of different tolerogenic mechanisms. In fact our preliminary data suggests that $Foxp3^+$ Treg cells may play a more important role in transplantation tolerance than in tolerance induced to allergens – something that still requires further elucidation.

Our data also suggests it is likely that in sensitized mice the antibody treatment has an impact exclusively on the late response (mediated by Th2 and NKT cells), without preventing the early response mediated by mast cell degranulation in response to their surface IgE cross-linking, as the allergen-specific IgG1 and IgE titers remain high in treated mice. But even without targeting early mast cell degranulation, the MAb treatment is likely to lead to a putative long-term benefit given the importance of the Th2-mediated response for the persistence of the inflammatory changes associated with airways remodeling and chronic manifestations of the disease [317]. This issue will require confirmation in chronic models of disease.

The antigen-specificity of Treg cell-mediated tolerance has been a controversial issue [38, 141]. We show that effective tolerance induction requires the presence of the appropriate antigen at the time of CD4 blockade leading to antigen-specific tolerance. In these experiments we waited 50 days following initial sensitization to minimize the amount of antigen still present in the animal at the time of the tolerogenic treatment. These data also established that MAbs administered at day 50 (Figure 3) were not contributing significantly to the prevention of the disease by being in circulation at the time of intranasal challenge, since no beneficial effect is observed in animals treated with the same antibody dose together

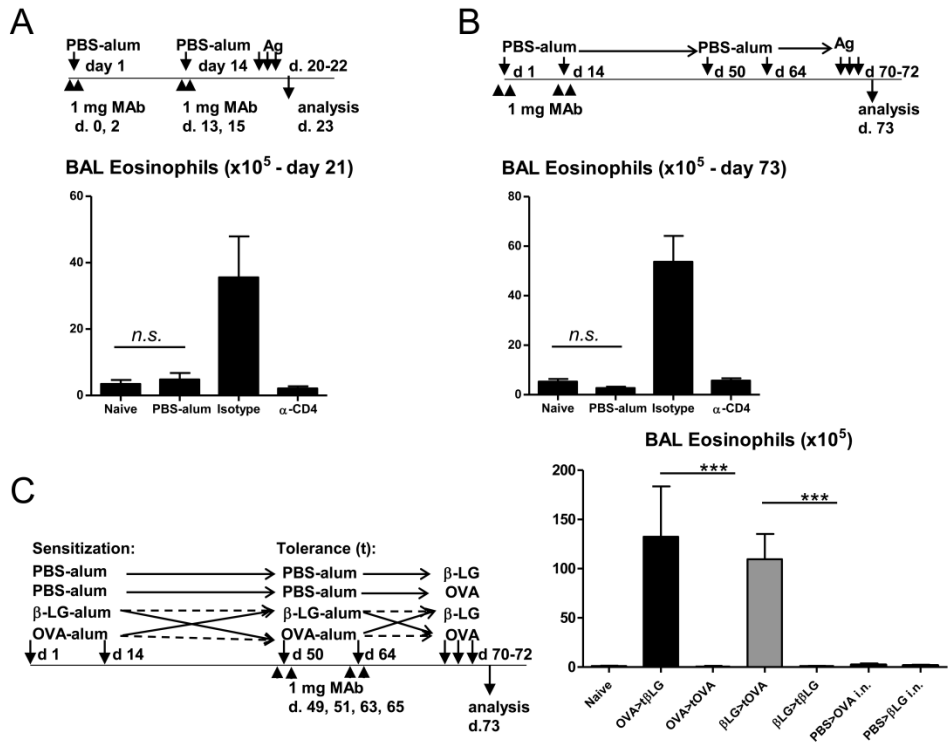
with an irrelevant antigen. Importantly, most previous studies addressing the putative tolerance-inducing potential of monoclonal antibodies, namely anti-CD4 [325], did not address the antigen-specificity of the phenomenon or the immune competence of treated mice.

Several MAbs have been recently used as immune modulators in a wide range of diseases, including allergy [328]. Anti-CD4 MAbs have been evaluated both in pre-clinical non-human primate models of transplantation and autoimmunity, as well as in clinical studies [323, 329, 330]. Their therapeutic effectiveness was modest, short-term, and likely to be a consequence of transient immunosuppression and not tolerance. With hindsight those unimpressive results are not surprising due to technical details related with dosing and the MAb characteristics. The clinical trials have used mouse or chimeric MAb that elicited immune responses leading to their rapid clearance [331]. In addition, most of those studies, including a clinical trial in human asthma (with the depleting anti-CD4 MAb keliximab) [323], did not take advantage of non-depleting anti-CD4 MAbs. Therefore, and as a consequence of the adverse side effects associated with depleting reagents, it was not possible to attain a neutralizing dose of anti-CD4 know to be the most effective for tolerance induction. At this time, particularly given the promising results with non-depleting anti-CD3 in early onset diabetes patients [314], the past experience of anti-CD4 in human patients should be reassessed in face of current knowledge. On this note, a new non-depleting and less-immunogenic humanized anti-CD4 MAb has been recently engineered, and its safety evaluated in human volunteers [332].

Our results suggest the specific targeting of CD4 T cells in allergic airways disease can have a potent effect in achieving long-term protection

from subsequent inflammatory changes induced by the same antigens. It remains to be established whether similar effects can be achieved in chronic allergic airways disease.

Supplementary Figures



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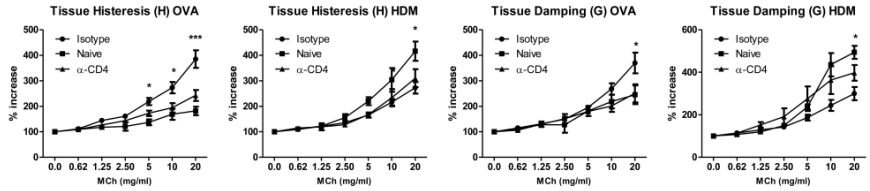
Supplementary Figure 1. Prevention of allergic sensitization with anti-CD4 MAbs. Female BALB/c mice were sensitized with 20 µg OVA-alum i.p. and challenged with 50 µg OVA in saline i.n. on the indicated days. Some animals were treated with 1 mg anti-CD4 or an isotype control i.p. as shown. Naive mice, not subjected to any intervention, were also studied as a control group and compared with mice injected with adjuvant in the absence of antigen at the time of sensitization. (A) Cellular composition of the BAL of mice treated with anti-CD4 at the time of sensitization. (B) Cellular composition of the BAL of mice treated with anti-CD4 at the time of initial sensitization, but subjected to additional sensitization at a subsequent time. (C) Cellular composition of the BAL of sensitized mice treated with anti-CD4.

Data related to figure 1



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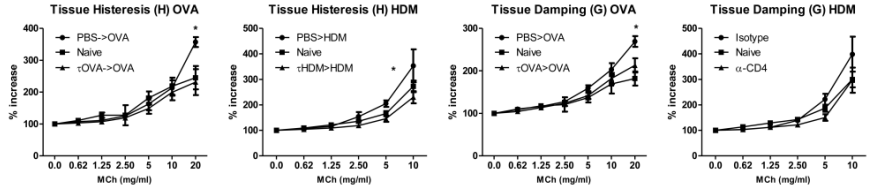

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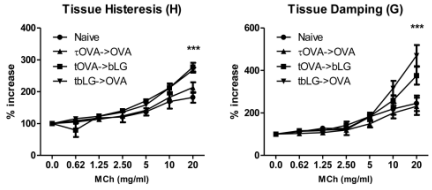

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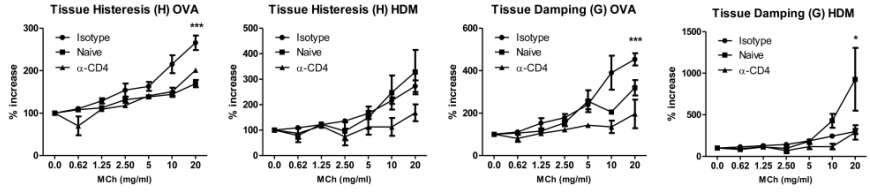

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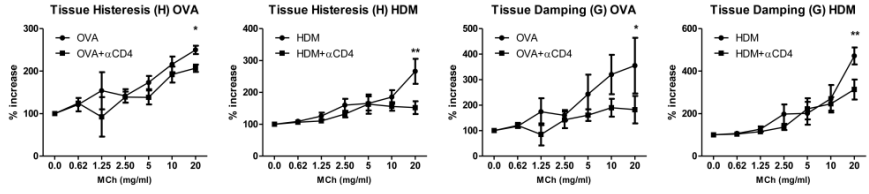
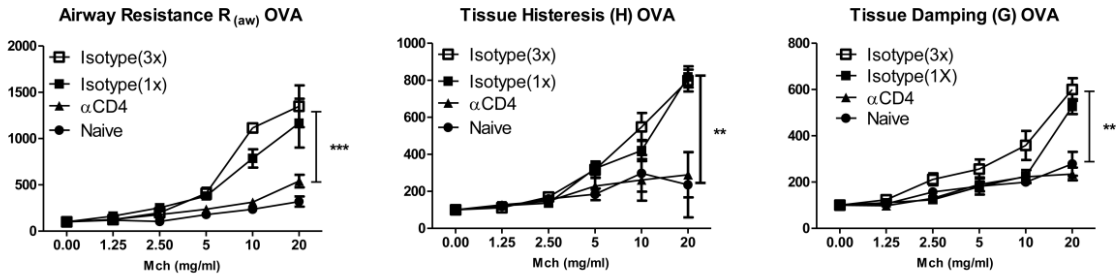
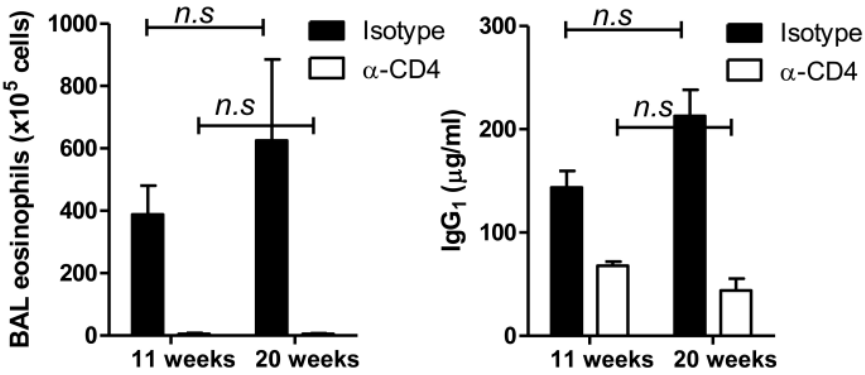


Supplementary Figure 2. Invasive measurement of respiratory mechanics. Data showing the impact of anti-CD4 treatment in tissue elastance and tissue damping in response to increasing doses of inhaled MCh. These graphs complement the data on airway resistance represented in the main figures 1 to 5.



Supplementary Figure 3. Induction of AHR following i.n. exposure to the antigen. Female BALB/c mice were sensitized with two shots of 20 µg OVA-alum i.p. 14 days apart, and challenged with 50 µg OVA in saline i.n. for three consecutive days (day 20-22), or just on day 20. Invasive measurement of respiratory mechanics was performed on the following day in presence of increasing doses of inhaled Mch. Both groups of mice, subjected to a single or three challenges with i.n. antigen, displayed similar levels of AHR (n=6, ** P<0.01, *** P<0.001).



Supplementary Figure 4.. Allergic airways disease in mice with different age. Female BALB/c mice were sensitized with 20 µg OVA-alum i.p. and challenged with 50 µg OVA in saline i.n. as indicated in Figure 1. Some animals were treated with 1 mg anti-CD4 or an isotype control i.p. at the time of sensitization. Mice with 11 or 20 weeks of age were used. No significant differences between mice of different ages.

Adjuvant facilitates anti-CD4 mediated immune tolerance to recombinant factor VIII in haemophilia by a Foxp3-independent mechanism that relies on IL-10

Introduction

For many patients with severe hemophilia, replacement therapy with recombinant clotting factors represents an immune challenge with non-self proteins thus resulting in an immune response. As a consequence, immunoglobulins targeting infused clotting factors can lead to their inhibition (those immunoglobulins are termed inhibitors)[333]. Several approaches for immune tolerance induction (ITI), leading to the control of the production of clotting factor inhibitors, are currently used in hemophilia patients[334]. However, the success of ITI is not absolute and currently, at a time when viral contaminants in therapeutic products are a problem of the past, inhibitors became a major complication for hemophilia treatment. This has led to an increasing interest in developing new protocols for the induction of tolerance to therapeutically administered clotting factors[335]. Recent clinical trials have explored the putative higher tolerogenicity of factor VIII (FVIII) coupled with von Willebrand Factor (vWF), or B cell depletion with an anti-CD20 monoclonal antibody (MAb) rituximab[336, 337]. The outcome of those trials has shown, at the most, a modest improvement in ITI efficacy. Consequently, those ITI approaches were not generally adopted and the development of better strategies for ITI remains an unmet medical need[338].

In recent years several strategies have been developed to promote immune tolerance towards alloantigens. Several studies have demonstrated that non-depleting monoclonal antibodies (MAbs) targeting T cell surface molecules are effective in achieving immune tolerance in transplantation and autoimmunity[148, 339]. There is, however, a contrast between the efficacy in achieving long-term tolerance with MAbs such as CD4 or CD40L administered at the time of transplantation in pre-clinical studies[140, 141, 340], with a transient effect observed when the same reagents are used to induce tolerance to human recombinant factor VIII (hFVIII) or factor IX (hFIX) in mice[341, 342]. Remarkably, when the recombinant clotting factors are expressed in tissues, for instance following viral transduction, tolerance induction appears to be easier to achieve[343].

These observations prompted us to investigate whether the difference between both outcomes is a consequence of differences in the way the antigen is presented to T cells for tolerance induction. It is plausible that efficient imposition of tolerance *in vivo*, would require an impact on a large proportion of the antigen-specific T cells, or stronger co-stimulation, something that can only be achieved in the presence of inflammation (namely in transplantation or viral transduction) or when an adjuvant is used.

We found that the most clinically relevant adjuvant – aluminium hydroxide (alum) – facilitates the induction of tolerance to hFVIII in mice. Surprisingly, the mechanism underlying tolerance induction appears to be different from antibody-induced transplantation tolerance: while non-depleting anti-CD4 induces Foxp3^{+} regulatory T cells that are indispensable for transplantation tolerance, we were able to induce tolerance to foreign

proteins in Foxp3-deficient mice. On the contrary, tolerance induction is critically dependent of IL-10, as it cannot be established in IL-$10^{-/-}$ mice.

Adjuvants can therefore have two opposing roles: they can contribute to boost immune responses and to overcome natural tolerance, but they can be instrumental in facilitating therapeutically induced tolerance when in presence of appropriate tolerance-inducing reagents.

Materials and methods

Animals. BALB/c, C57Bl/6, B6.IL-$10^{-/-}$, B6.Rag$2^{-/-}$, and DO11.10.Rag$2^{-/-}$, mice were bred and maintained under specific pathogen-free (SPF) facilities at the Instituto Gulbenkian de Ciência. Hemophilia-A mice (HemA) with a targeted disruption of exon 16 in the FVIII gene were initially generated by Kazazian and colleagues[344]. HemA mice backcrossed into C57Bl/6 and BALB/c background were generously provided by Professor David Lillycrap (Queen's University, Kingston, Canada) and were maintained under SPF facilities at the Instituto de Medicina Molecular. The T/B monoclonal mice (T-Bmc, 17/9 DO11.10.RAG$1^{-/-}$) bear monoclonal populations of T and B lymphocytes specific for chicken ovalbumin (OVA) 323–339 and HA of influenza virus, respectively[345]. These mice were kept under SPF conditions at the Skirball Institute Central Animal Facility, New York University Medical Center. All procedures involving animals were approved by the Direção Geral de Veterinária (Lisbon) or New York University School of Medicine Institutional Animal Care and Use Committee (New York).

Animals were exposed to 1U hFVIII (Kogenate, Bayer, Germany), or to 20 µg OVA (Sigma, St Louis, USA) previously run through a DetoxyGel column (Pierce, Rockford, USA). In some experiments the antigens were adsorbed in 2.0 mg of endotoxin-free aluminum hydroxide (Alu-gel-S, Serva, Heidelberg, Germany). In experiments involving alum the antigens were administered i.p. We confirmed that induction of inhibitors was similar through the i.p. or i.v. route.

Monoclonal antibodies. Non-depleting anti-CD4 (YTS177.9)[153], the isotype control (anti-canine CD4; YKIX302), anti-CD25 (PC61), anti-IL-10R (1B1.1), and anti-TGFβ (1D11) MAbs were produced in house using Integra CL1000 flasks (IBS, Chur, Switzerland), and purified from culture supernatants by 50% ammonium sulfate precipitation, dialyzed against PBS, and the purity checked by native and SDS gel electrophoresis. YTS177.9 and YKIX302 hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Quantification of immunoglobulins and cytokines. Serum titers of hFVIII-specific, OVA-specific, and HA-specific IgG1 were measured by ELISA using the following: IgG1-HRP (SouthernBiotech, Birmingham, USA) with anti-hFVIII IgG1 standard (Abcam, Cambridge, UK), anti-OVA IgG1 standard (Serotec, Oxford, UK) and anti-HA IgG1 standard (Sigma, St Louis, USA). The ELISA assay for IL-10 measurement was performed using a kit (Peprotech, London, UK) and according to the manufacturer's instructions.

Flow cytometry. Cells were stained with CD25-AlexaFluor488 (PC61; produced and conjugated in house), CD25-PE-Cy7 (PC61.5; eBioscience), Foxp3-APC (FJK-16s; eBioscience), CD3-PE-Cy7 (145-2C11; eBioscience), CD4-PerCp (RM4-5; BD Pharmigen), and the DO11.10 TCR-specific KJ1-26

MAb conjugated with PE (BD Pharmigen) in PBS containing 0,01% NaN3, 2% fetal bovine serum (FBS, Gibco) and 10 µg/ml Fc receptor blocking (2.4G2; produced and purified in house). Six color analyses were performed using a LSR Fortessa or a FACSCanto (BD Bioscience) with dual laser (488nm and 633nm) excitation. The analysis gate was set on the forward and side scatters to eliminate cell debris and dead cells.

Bethesda assay. Inhibitory FVIII Abs were measured using the Bethesda assay and reported as Bethesda units/mL as previously described[346]. Briefly, mouse plasma was serially diluted in dilution buffer (Technochrom® FVIII:C, Technoclone, Austria) such that the residual FVIII:C for each sample was between 25% and 75%, mixed 1:1 with pooled normal human plasma (Factor VIII Inhibitor reagent Kit- Bethesda Units), and incubated for 2 hours at 37°C. The remaining FVIII activity was quantified using a chromogenic assay (Technochrom). For all samples, to reduce error in the assay and inconsistency between samples, the reported inhibitor titer in BU/mL was calculated from the dilution of plasma that yielded a residual FVIII:C of approximately 50%. By definition 1 BU/mL is defined as the dilution of plasma containing FVIII inhibitory activity that results in 50% inhibition of FVIII activity (FVIII:C).

Statistical analysis. Statistical significance was determined using the two-tailed non-parametric Mann-Whitney U test and the Spearman test for correlation analysis. Statistical analysis was performed using Prism 4.0 (GraphPad, San Diego, USA) and values of $P<0.05$ were deemed significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Results

hFVIII alum increases the tolerogenicity of anti-CD4 MAb.

Alum has been used as an adjuvant in the formulation of human vaccines since 1926, having been administered to millions of humans to boost immunization[347, 348]. In order to exclude a putative strain influence in the outcome we treated BALB/c and C57Bl/6 mice with hFVIII or hFVIII-alum together with a non-depleting anti-CD4 MAb at the indicated time points (**Figure 1a**). Serum levels of anti-hFVIII IgG1 were measured following additional administrations of hFVIII. Our results confirmed that anti-CD4 treatment only has a moderate impact in preventing the generation of inhibitor antibodies to hFVIII (**Figure 1b, c**). However, when hFVIII-alum was used at the time of anti-CD4 treatment the concentration of hFVIII-specific antibodies remained significantly reduced even following multiple administrations of hFVIII up to 110 days following the initial treatment. Note that hFVIII-alum is, as anticipated, highly immunogenic due to the adjuvant properties of alum, leading to very high levels of anti-hFVIII immunoglobulins when used in the absence of anti-CD4.

Next we investigated whether the route of administration of hFVIII-alum could influence the tolerance induction. C57Bl/6 mice were therefore exposed to hFVIII-alum i.p. (as described above), or through the intramuscular or subcutaneous route. In addition, administration of anti-CD4 and hFVIII was performed i.v. (**Figure 1d**). We found that tolerance

could be induced regardless of the route of administration of hFVIII-alum and anti-CD4.

The "depot effect" has been regarded as a possible mechanism by which alum exerts its adjuvanticity[349]. According to this effect, adsorption of hFVIII in Alum would confer a slow release and consequently an increased half-life of hFVIII *in vivo* thus providing stable and long-term antigen presentation. This hypothesis is especially relevant as it has been described that sustained release of peptides, by an implanted osmotic pump, can lead to immune tolerance[350]. We addressed this issue by repeatedly injecting hFVIII every other day for two weeks in presence of anti-CD4 MAb. We found that animals exposed to multiple administrations of hFVIII+anti-CD4 still produced higher titers of anti-hFVIII IgG1 than animals treated with hFVIII-alum+anti-CD4 (**Figure 1e**). Our data show alum is potentiating the tolerogenicity of anti-CD4 MAb by a mechanism independent of the depot effect.

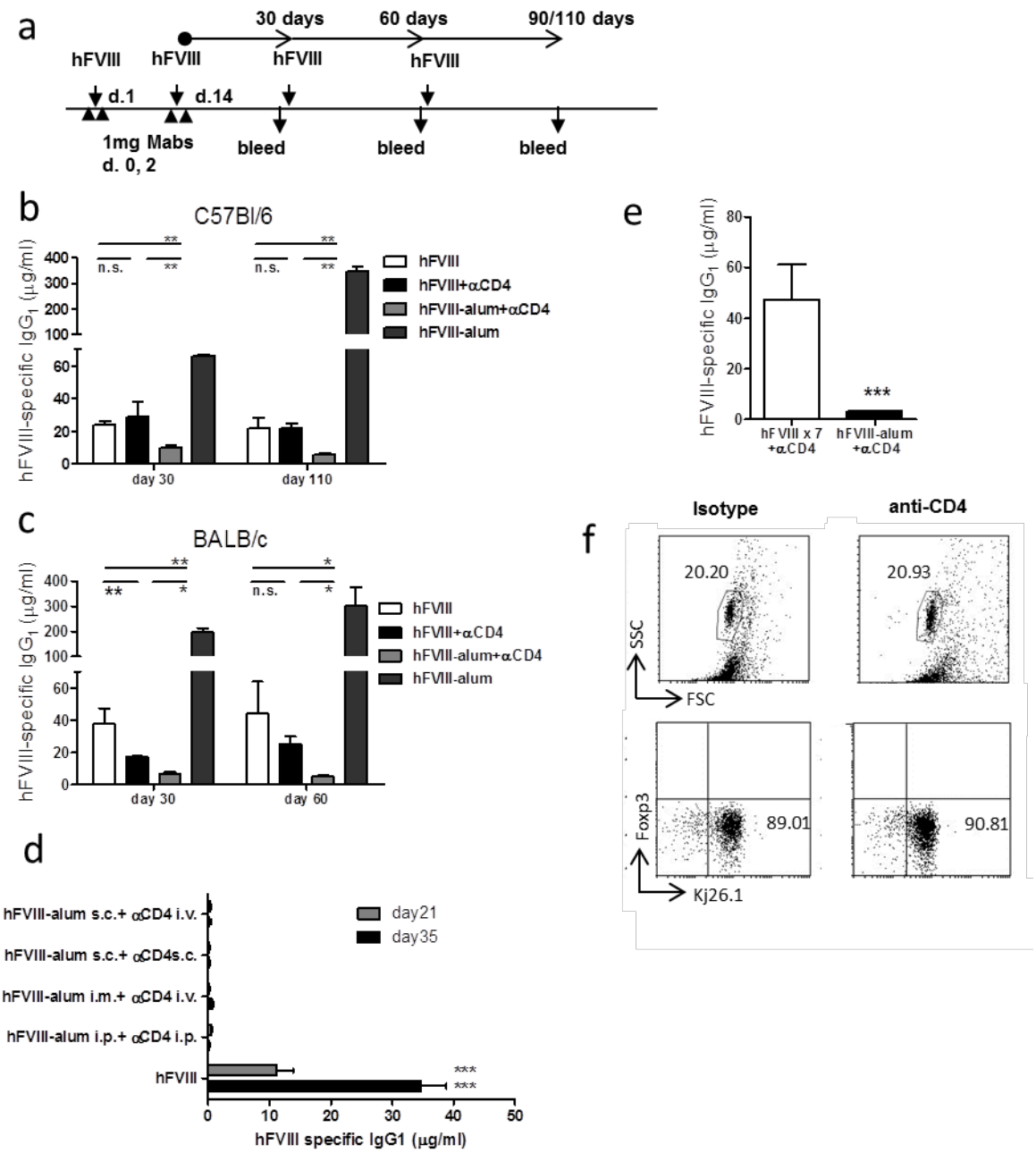


Figure 1. *hFVIII-alum increases the tolerogenicity of anti-CD4 Mab.* (A) C57Bl/6 and Balb/c mice were treated with 1 U hFVIII or HFVIII-alum and 2 x 1 mg non-depleting anti-CD4 (or isotype control) on the indicated days, followed by subsequent administrations of 1 U hFVIII as represented. The presence of serum

hFVIII-specific Ig was investigated at days 30, 60, and 90 or 110 following last administration of anti-CD4. (B) Serum concentration of hFVIII-specific IgG1 in C57Bl/6 mice (n=6; **: $p<0.01$), and (C) in Balb/c mice (n=6; *: $p<0.05$; **: $p<0.01$). (D) hFVIII-alum and anti-CD4 were administered according to the schedule represented in (A) through different routes (as indicated in the label). One group of mice remained untreated. All mice were then exposed to hFVIII and serum titres of anti-hFVIII were quantified. Animals that received hFVIII-alum together with anti-CD4 remained tolerant regardless the administration route (n=6; $p<0.001$). (E) Persistent serum levels of hFVIII did not prevent the generation of hFVIII-specific IgG1 in presence of anti-CD4. C57Bl/6 mice were treated with anti-CD4 MAbs in the days indicated above. Some mice were treated with 1 U hFVIII every other day for two weeks. We found that animals exposed to multiple administrations of hFVIII still produced higher titers of anti-hFVIII IgG1 than animals treated with hFVIII-alum (n=6; $p<0.001$). (F) Administration of the non-depleting anti-CD4 MAb (YTS177.9) does not lead to CD4 T cell depletion. DO11.10.Rag$^{-/-}$ mice were treated with 2 x 1 mg YTS177.9 and splenocytes were examined by flow cytometry for evidence of T cell depletion at day 7 (n=5; *p*, non-significant).

Although the anti-CD4 MAb has a non-depleting isotype, we confirmed it does not lead to direct T cell depletion *in vivo* by treating DO11.10.Rag2$^{-/-}$ mice with anti-CD4. This way we could circumvent the coating of the CD4 molecule, being able to accurately quantify CD4^{+} T cells by staining their transgenic TCR (identified with KJ26.1 MAbs). We found that the frequency of CD4+ splenocytes at day 7 following anti-CD4 treatment remained unchanged (**Figure 1f**).

Tolerance induction requires IL-10 and is independent of Foxp3^{+} Treg cells.

We and others have shown that transplantation tolerance can be induced with non-depleting anti-CD4 MAb, in a process that is Foxp3^{+} Treg cell-mediated and TGFβ-dependent[39, 159, 340, 351, 352]. In some conditions, IL-10 appears to play a role in transplantation tolerance[351,

353]. In order to study the mechanism by which anti-CD4 MAb induces tolerance in hFVIII-alum treated animals, we used MAbs to block TGF-β or IL-10R at the time of treatment. In addition, another group of animals was treated with a depleting anti-CD25 MAb prior to treatment. We found IL-10R-blockade during the initial two weeks had a dramatic effect in preventing tolerance induction (**Figure 2a**). However, only a modest increase in the concentration of anti-hFVIII IgG1 was observed in mice subjected to depletion of $CD25^+$ T cells or to neutralization of TGF-β.

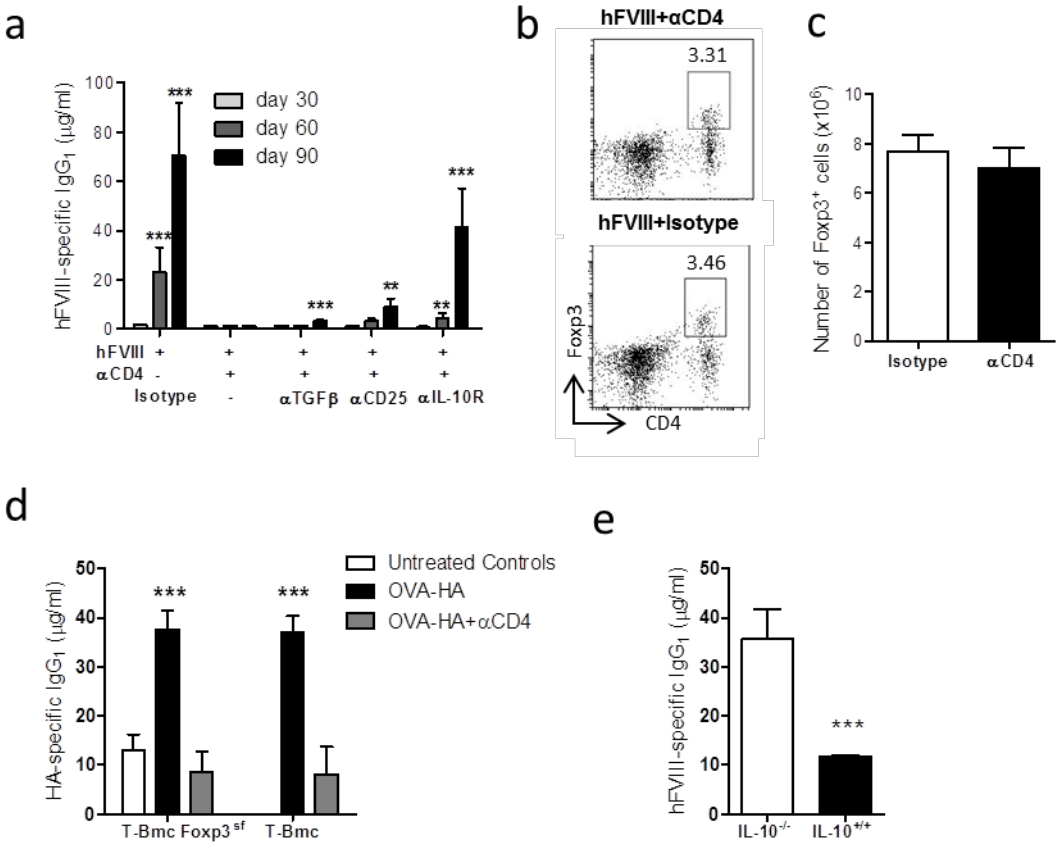


Figure 2. *Tolerance induction requires IL-10 and is independent of $Foxp3^+$ Treg cells.* (A) Mice were treated with hFVIII-alum and anti-CD4 as described in Figure 1A. Different groups were also treated with the indicated MAbs. All mice were exposed to 1 U hFVIII on days 30 and 60. Serum concentrations of hFVIII-specific

IgG1 were determined on days 30, 60, and 90 (n=5; **: p<0.01; ***: p<0.001). (B) Representative dot-plots and (C) quantification of splenic Foxp3$^+$ T cells from animals treated with anti-CD4 MAb or an isotype control, at day 90 (n=5; p, non-significant). (D) T/B monoclonal mice (T-Bmc) T-Bmc Foxp3-deficient mice (T-Bmc FoxP3sf), were immunized on days 1 and 14 with OVA-HA-alum in the presence of non-depleting anti-CD4 or an isotype control. Serum HA-specific IgG1 was determined on day 24 (n=5; p<0.001). (E) IL-10$^{-/-}$ and IL-10$^{+/+}$ mice were treated with hFVIII-alum and anti-CD4 MAbs as described above. The serum concentration of hFVIII-specific IgG1 was quantified on day 90 (n=6; p<0.001). (A-E) Data are representative of two independent experiments.

These data suggest Foxp3$^+$ Treg cells may only have a minor role in mediating tolerance in our model. In fact, no differences were found in percentages or absolute numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells in the spleen of animals treated with hFVIII-alum + anti-CD4 when compared to the positive group (hFVIII-alum + isotype) 90 days after the induction of tolerance (**Figure 2b and 2c**).

Given the fact that experimental mice have a polyclonal T cell repertoire, putative changes in the frequency of antigen-specific Treg cells may be beyond the limit of detection. In addition, anti-CD25 depletion with PC61 is known to spare a significant number of Foxp3$^+$ Treg cells[354]. We therefore used Foxp3-deficient mice to directly assess if tolerance to a foreign protein could be induced in the absence of Foxp3$^+$ Treg cells. Since Foxp3-deficiency leads to severe autoimmunity we had to perform this experiment in mice devoid of autorreactive T cells. We used T/B Foxp3^{sf} monoclonal mice (T-Bmc FoxP3^{sf}), characterized by monoclonal populations of T and B lymphocytes specific, respectively, for chicken ovalbumin 323–339 (OVA) and heme-aglutinin (HA) of influenza virus[345]. As a consequence, we had to use a cross-linked protein of OVA-HA as a model antigen instead of hFVIII. Treatment of T-Bmc mice with OVA-HA-alum and anti-CD4 MAb

was equally effective in the presence or absence of functional Foxp3 (**Figure 2d**).

To further confirm the key role of IL-10 in inducing tolerance to hFVIII, we treated IL10$^{-/-}$ mice with hFVIII-alum + anti-CD4 MAb. We confirmed that it was not possible to prevent generation of anti-hFVIII IgG1 in IL10$^{-/-}$ mice by treatment with hFVIII-alum + anti-CD4 (**Figure 2e**).

Taken together, our data show that induction of tolerance to hFVIII-alum in the presence of anti-CD4 MAb is IL-10-dependent.

Non-depleting anti-CD4 induces long-term prevention from inhibitor formation in hemophilia A mice.

To determine if treatment with anti-CD4 could prevent the formation of FVIII inhibitors in hemophilia, we used mice deficient in FVIII (hemophilia A mice, HemA). We treated C57Bl/6 HemA mice with hFVIII-alum and anti-CD4 MAb as described in Figure 1a. Our data show that HemA mice treated with anti-CD4 exhibited low serum concentration of anti-hFVIII IgG1 for 90 days, even after multiple exposures to hFVIII (**Figure 3a**). In addition, we used the Bethesda Assay to measure the titer of FVIII inhibitors at day 90. We found that anti-CD4 treatment successfully prevented the formation of inhibitors in HemA mice (**Figure 3b and 3c**). Similar results were obtained with BALB/c HemA mice (**Figure 3d and 3e**). We also found that in addition to hFVIII-specific IgG1, the hFVIII-exposed HemA mice produced high titres of hFVIII-specific IgG2a and IgG2b, unless mice were treated with anti-CD4 MAb (Supplemental Figure 1). While C57Bl/6 HemA mice predominantly produced IgG1 and IgG2a, BALB/c HemA mice produced lower titres of

hFVIII-specific IgG2a but higher titres of IgG2b. Our findings on the different isotype preferences by the two strains of HemA mice are consistent with previous reports[355].

We confirmed that tolerance induction could also be achieved in HemA mice when hFVIII-alum was administered s.c. and the anti-CD4 MAb i.v. (**Figure 3f and 3g**).

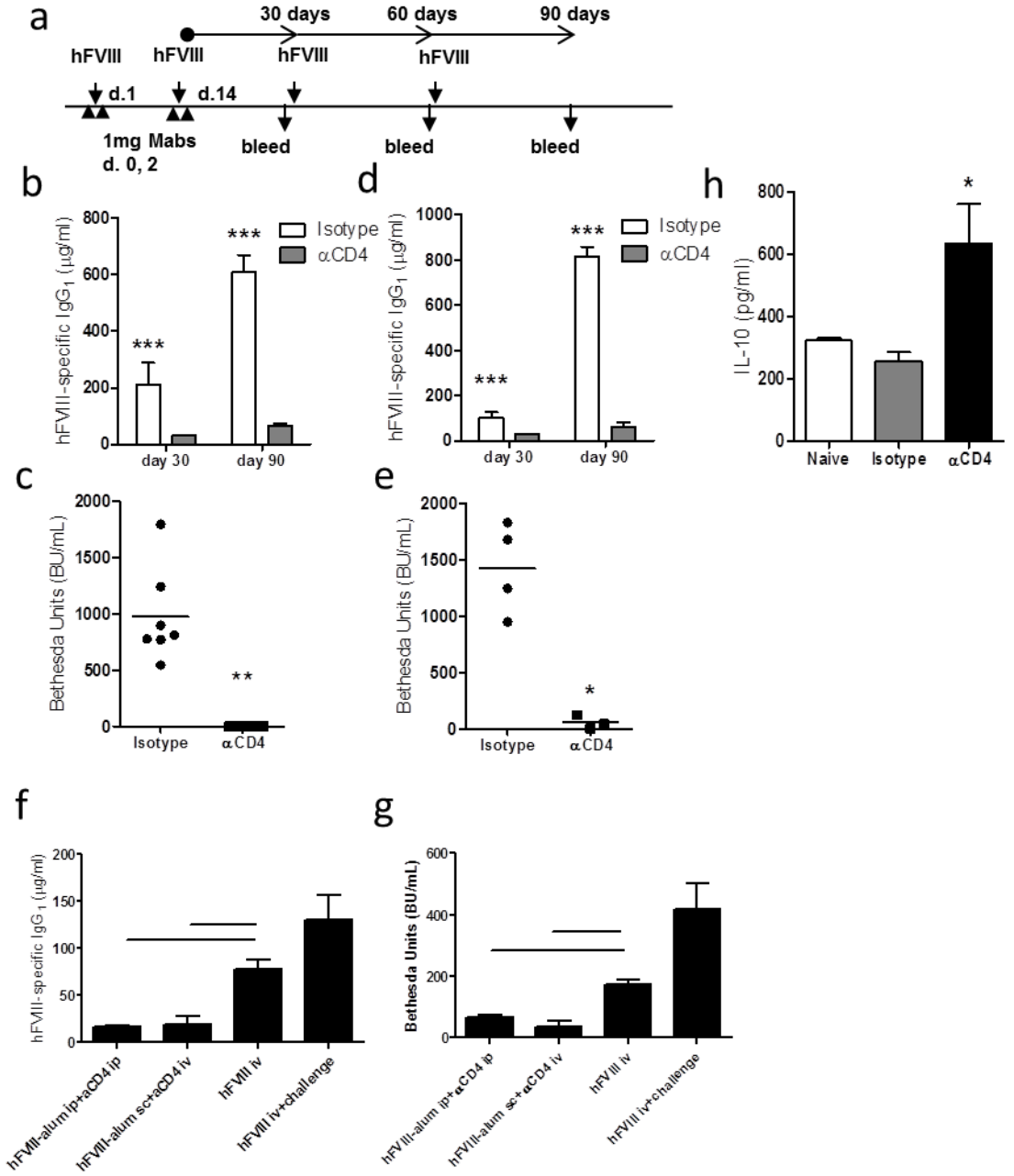


Figure 3. *Non-depleting anti-CD4 induces long-term protection from inhibitor formation in hemophilia-A mice.* (A) HemA mice were treated with 1 U hFVIII or HFVIII-alum and 2 x 1 mg non-depleting anti-CD4 (or isotype control) on the indicated days, followed by subsequent administrations of 1 U hFVIII as represented. The presence of serum hFVIII-specific Ig was investigated at days 30,

60, and 90 following last administration of anti-CD4 . (B) Quantification of serum hFVIII-specific IgG1 from C57Bl/6 Hem-A mice at day 30 and at day 90, following multiple exposures to hFVIII (n=8; $p<0.001$). (C) Quantification of FVIII inhibitors from the serum of C57Bl/6 mice at day 90 (n=8; $p<0.01$). (D) Serum hFVIII-specific IgG1 from BALB/c Hem-A mice (n=4; $p<0.01$). (E) Quantification of hFVIII inhibitors from BALB/c Hem-A mice (n=4; $p<0.05$). (F) C57Bl/6 HemA mice were treated with hFVIII-alum (either i.p. or s.c.) and anti-CD4 (either i.p. or i.v., respectively), on days 1 and 14. One control group remained untreated, while another was treated with hFVIII + isotype control. Subsequent challenges were done by i.v. administration of hFVIII, in a total of 4 injections, given twice weekly starting on day 21. Quantification of the anti-hFVIII IgG1 levels and (G) Bethesda Units, measured one week after the final exposure to hFVIII (n=6; $p<0.05$). (H) Quantification of IL-10 concentration in supernatants of splenocytes from C57Bl/6 Hem-A mice, collected at day 7 following initial treatment with HFVIII-alum + anti-CD4. The splenocytes were stimulated with anti-CD3 for three days (n=3; $p<0.05$). (A-E) Data are representative of two independent experiments.

We also found that splenocytes collected from HemA mice treated with hFVIII-alum + anti-CD4 when stimulated *in vitro* in presence of anti-CD3 were more prone to produce IL-10 than splenocytes from naïve non-manipulated mice, or mice treated with an isotype control (**Figure 3h**).

Tolerant mice remain immune competent.

Although the anti-CD4 Mab clone has a non-depleting isotype (i.e. it does not directly lead to T cell lysis), we confirmed that mice treated with anti-CD4 remained immune competent. We used C57Bl/6 mice treated with anti-CD4 as described in Figure 1, but using hFVIII-alum or OVA-alum as the "tolerizing antigen" administered at the time of Mab treatment. We waited five weeks to minimize the potential persistence of antigen or anti-CD4. At day 50 and 64, the mice were exposed to the same or to the other antigen (**Figure 4a**). We found that treatment with anti-CD4

Mab prevented subsequent immunization to the same antigen without suppressing immune responses to the other antigen (**Figure 4b and 4c**). In other words, mice "tolerized" with OVA-alum could respond to subsequent immunization with FVIII-alum, but did not respond to OVA-alum. Conversely, mice "tolerized" with FVIII-alum did not respond to a later challenge with FVIII-alum, but were fully competent to produce antibodies following immunization with OVA-alum.

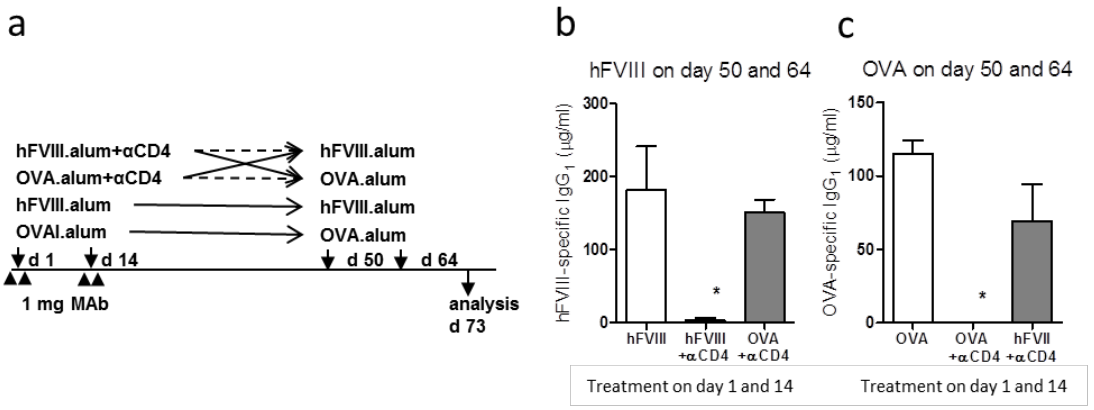


Figure 4. *Non-depleting anti-CD4-treated mice remain immune competent.* (A) C57Bl/6 mice were treated as described previously with anti-CD4, but using FVIII-alum or OVA-alum as the "tolerizing antigen" administered at the time of Mab treatment. Five weeks following the last MAb infusion the mice were exposed to the same or to the other antigen. Control groups were not treated with anti-CD4. (B) Some mice were immunized with hFVIII-alum on days 50 and 64, and serum hFVIII-specific IgG1 was quantified at day 73. We found that unlike mice initially treated with OVA-alum + anti-CD4, mice treated with hFVIII-alum + anti-CD4 remained unresponsive to the subsequent immunization with hFVIII-alum (n=4; $p<0.05$). (C) Other mice were immunized at day 50 and 64 with OVA-alum, and serum OVA-specific IgG1 was quantified on day 73. Animals initially treated with hFVIII-alum + anti-CD4 remained competent to respond to immunization with OVA-alum, unlike mice initially treated with OVA-alum + anti-CD4 that remained tolerant to OVA (n=4; $p<0.05$). Data are representative of two independent experiments.

These results show that treatment with anti-CD4 does not induce global immune suppression, but rather antigen-specific unresponsiveness.

Anti-CD4 MAb is effective in inducing tolerance following sensitization

Unlike autoimmunity or allergy, in hemophilia there is an opportunity to induce tolerance in advance of immune sensitization, as the first exposure to the antigens is controlled by the clinician. However, since many hemophilia patients do not develop inhibitors, ITI strategies are often required when the patient is already sensitized. Previous studies suggested that CD4-blockade may be effective in sensitized hosts[46]. But hFVIII-specific IgG produced at the time of sensitization, given their long half-life, may obscure the eventual effectiveness of tolerance induction at a later time.

To overcome this limitation, and since it is not feasible, in mice, to remove pre-existing FVIII-specific IgG by plasmapheresis, we developed an experimental setting to address the role of CD4-blockade in pre-sensitized cells in the absence of preformed FVIII-specific IgG (**Figure 5a**).

In brief, C57Bl/6 mice were immunized with hFVIII-alum at day 1 and 14, and sacrificed at day 30. This immunization protocol leads to high concentration of hFVIII-specific IgG1 (198.0 ± 14.7 μg/ml). We collected and pooled T and B cells from spleen, pooled lymph nodes, and bone marrow. The lymphocytes were then adoptively transferred i.v. into congenic $Rag2^{-/-}$ or C57Bl/6 recipients. The recipient mice were then treated with hFVIII-alum + anti-CD4 Mab, and exposed to additional challenges with hFVIII (**Figure 5a**).

Using $Rag2^{-/-}$ recipients we confirmed that the adoptively transferred primed T and B cells were sufficient to produce anti-hFVIII IgG1 (**Figure 5b**).

However, treatment with anti-CD4 was effective in preventing the production of hFVIII-specific IgG1 by those primed cells (**Figure 5b**).

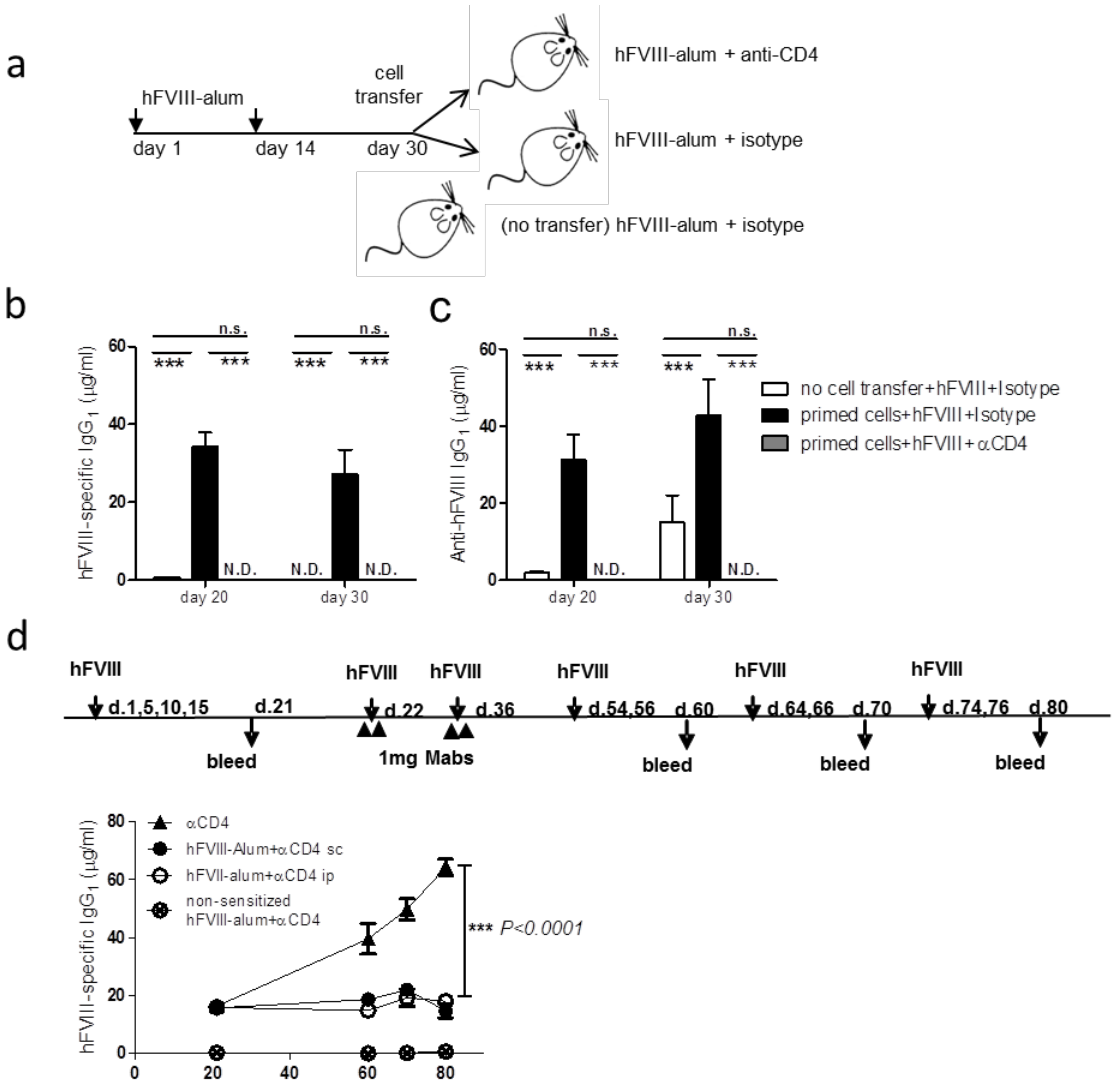


Figure 5. *Anti-CD4 Mab is effective in inducing tolerance following sensitization.* (A) C57Bl/6 mice were sensitized with hFVIII-alum at day 1 and 14, and sacrificed at day 30. Leukocytes from spleen, pooled lymph nodes, and bone marrow were collected and adoptively transferred i.v. into congenic $Rag2^{-/-}$ or C57Bl/6 recipients. The recipient mice were then treated with hFVIII-alum + anti-CD4 Mab (or an isotype control) on the day of cell transfer and 14 days later (consistent with protocol represented in Figure 1A), and all groups of mice were

subsequently challenged with 3x 1U hFVIII on days 10, 12, and 14 following the last administration of anti-CD4. (B) Quantification of serum hFVIII-specific IgG1 in $Rag2^{-/-}$ recipient mice. Animals that received primed T and B cells were competent to produce anti-hFVIII IgG1 (n=5; $p<0.001$), but antibody production was abrogated in mice treated with anti-CD4 (n=5; $p<0.001$; N.D., not detected). (C) Concentration of serum hFVIII-specific IgG1 in C57Bl/6 mice transferred with primed T and B cells. Animals adoptively transferred with primed cells produced higher levels of hFVIII-specific IgG1 than animals that did not received primed cells (n=5; $p<0.001$). Treatment with anti-CD4 prevented production of hFVIII-specific IgG1 by the primed lymphocytes (n=5; $p<0.001$; N.D., not detected). Data are representative of two independent experiments. (D) C57Bl/6 mice were sensitized with hFVIII on days 1, 5, 10, and 15, and treated with hFVIII-alum and anti-CD4 (either i.p. or s.c.) on days 35 and 50. Control mice received anti-CD4 without hFVIII. One control group had not been previously exposed to hFVIII. All mice received hFVIII i.v. on days 64, 66, 74, 76, 84, and 86. Quantification of anti-hFVIII IgG1 was performed on the indicated days. Mice treated with hFVIII-alum + anti-CD4 (regardless of the route of administration) were protected from production of anti-hFVIII IgG1 following subsequent exposure to hFVIII (n=6; $p<0.001$).

The adoptive cell transfer into C57Bl/6 mice showed that the adoptively transferred cells were indeed primed. Our data show that recipients of primed cells displayed a rapid and high magnitude secondary type immune response following exposure to hFVIII, when compared with control mice that did not receive primed cells, and that displayed a primary response (**Figure 5c**). We also observed that when the primed cells were transferred into C57Bl/6 mice, treatment with anti-CD4 Mab prevented production of anti-hFVIII IgG1 (**Figure 5c**).

We then investigated whether hFVIII-alum + anti-CD4 could protect mice sensitized to hFVIII from further production of FVIII-specific antibodies. We found that animals exposed to hFVIII when subsequently treated with hFVIII-alum (i.p. or s.c.) + anti-CD4 maintained the serum levels of FVIII-specific IgG1 in spite of repeated administrations of hFVIII (**Figure 5d**). The effect was not due to non-specific effects of anti-CD4, as

mice treated with anti-CD4 in the absence of hFVIII responded to subsequent exposure to hFVIII with the production of hFVIII-specific IgG1.

These results demonstrate that anti-CD4 Mab treatment is effective in controlling the response to hFVIII in pre-sensitized animals.

Discussion

Our results show that an adjuvant – alum – can enhance the ability of non-depleting anti-CD4 MAb to achieve tolerance to hFVIII. These results can appear counterintuitive as usually adjuvants, such as alum, are used to boost immunity namely leading to production of protective antibodies. It is likely that when tolerance is based on CD4-blockade it will be necessary that the majority of antigen-specific T cells will be exposed to the antigen during the time when the CD4-blocking MAb is present, and with optimal co-stimulation. In this regard, our results on the antigen-specificity of tolerance (Figure 4) also confirm that tolerance is imposed on the lymphocytes that are engaged in antigen recognition at the time of CD4-blockade: our data show that exposure to hFVIII-alum + anti-CD4 can prevent subsequent immune responses targeting hFVIII, while allowing successful immunization to an unrelated antigen (OVA). Similar results were obtained in the converse experiment: when tolerance is induced with OVA-alum + anti-CD4, the animals can produce antibodies targeting hFVIII, following exposure to the clotting factor. We can conclude that the initial exposure to hFVIII-alum is therefore critical to control the FVIII-specific T cell clones, but does not affect T cell clones with other specificities.

Given the previous reports showing a clear role of $Foxp3^+$ Treg cells in transplantation tolerance induced with anti-CD4[39, 340, 356], it was surprising to find that tolerance induction to proteins in alum could be achieved in the absence of Foxp3 (Figure 2). We have confirmed that indeed tolerance induction to skin grafts, induced following anti-CD4 MAb treatment, is dependent of $Foxp3^+$ Treg cells (data not shown). In the same fashion, several animal models of autoimmunity can be successfully

treated with anti-CD4, by a mechanism that appears to be Foxp3-dependent[357]. However, the systemic distribution a protein in alum appears to take advantage of an IL-10-dependent and Treg independent mechanism. This observation may explain the apparently contradictory results from transplantation studies using anti-CD4 for tolerance induction: while studies where a skin graft alone is used tolerance appears to be IL-10-independent and Treg dependent[158, 340]; but studies where donor specific transfusion and a skin graft are used appear to rely on both Treg cells and IL-10[351, 353], suggesting that systemic distribution of the antigen may recruit a IL-10-mediated tolerogenic mechanism. It remains to be established the reason for the absolute requirement of $Foxp3^+$ Treg cells in transplantation tolerance[356], while the same clone of non-depleting anti-CD4 MAb leads to Foxp3-independent immune tolerance in hemophilia.

By neutralizing IL-10R *in vivo* during the first two weeks of anti-CD4 treatment we could infer the participation of IL-10 in the mechanism leading to tolerance, but we could also establish the importance of that initial period for tolerance induction (Figure 2). This observation is consistent with the need to impose tolerance in antigen-specific cells during a window of time, after which the immune system regains the ability to respond to foreign antigens, as discussed above. In addition, by showing that repeated administrations of hFVIII (without alum) during those initial two weeks did not lead to long-term tolerance, as we observed when hFVIII-alum was used (Figure 1C), allowed us to exclude that tolerance facilitation by alum could be simply due to the “depot effect” leading to greater half-life of the antigen.

A usual concern in transplantation tolerance experiments is the influence of the genetic background in the results[229]. Some strains, namely C3H and CBA/Ca are known to be more permissive to the induction of transplantation tolerance than other strains like C57Bl/6 and BALB/c. In order to evaluate whether tolerance to hFVIII was strain-specific, we confirmed that long-term tolerance to hFVIII could be achieved in C57Bl/6 and BALB/c mice, and in mice from those two strains deficient in FVIII (HemA mice). When we used hemophilic mice we could directly document the physiological impact of FVIII inhibitors, not only by their quantification using the Bethesda assay but also by our subjective assessment of bleeding from those mice. Treatment with hFVIII-alum + anti-CD4 led to long-term protection from the generation of inhibitors in the two strains of hemophilic mice.

Several other MAbs targeting T cell co-receptors (namely anti-CD3) and co-stimulatory molecules have been shown effective in inducing immune tolerance in transplantation, autoimmunity, and allergic diseases[339, 358]. Recently it was reported that antibodies targeting CD3 can induce long-term tolerance to FVIII in hemophilic mice[359]. In addition, MAb targeting ICOS are effective in inducing long term tolerance to hFVIII in mice, following gene therapy[343]. Concerning non-depleting anti-CD4 there is, however, one special case of successful induction of long-term tolerance to foreign proteins in the absence of adjuvant – it is when the foreign protein is an immunoglobulin. In fact in some of the earliest demonstrations that non-depleting anti-CD4 can induce tolerance to foreign antigens, immunoglobulins (Ig) from different animal species were used as the tolerizing antigen[146, 147]. It is likely that the special tolerogenicity of foreign Ig compared with FVIII represents the fact that

antigen presenting cells can capture foreign Ig with specific Fc receptors for efficient presentation.

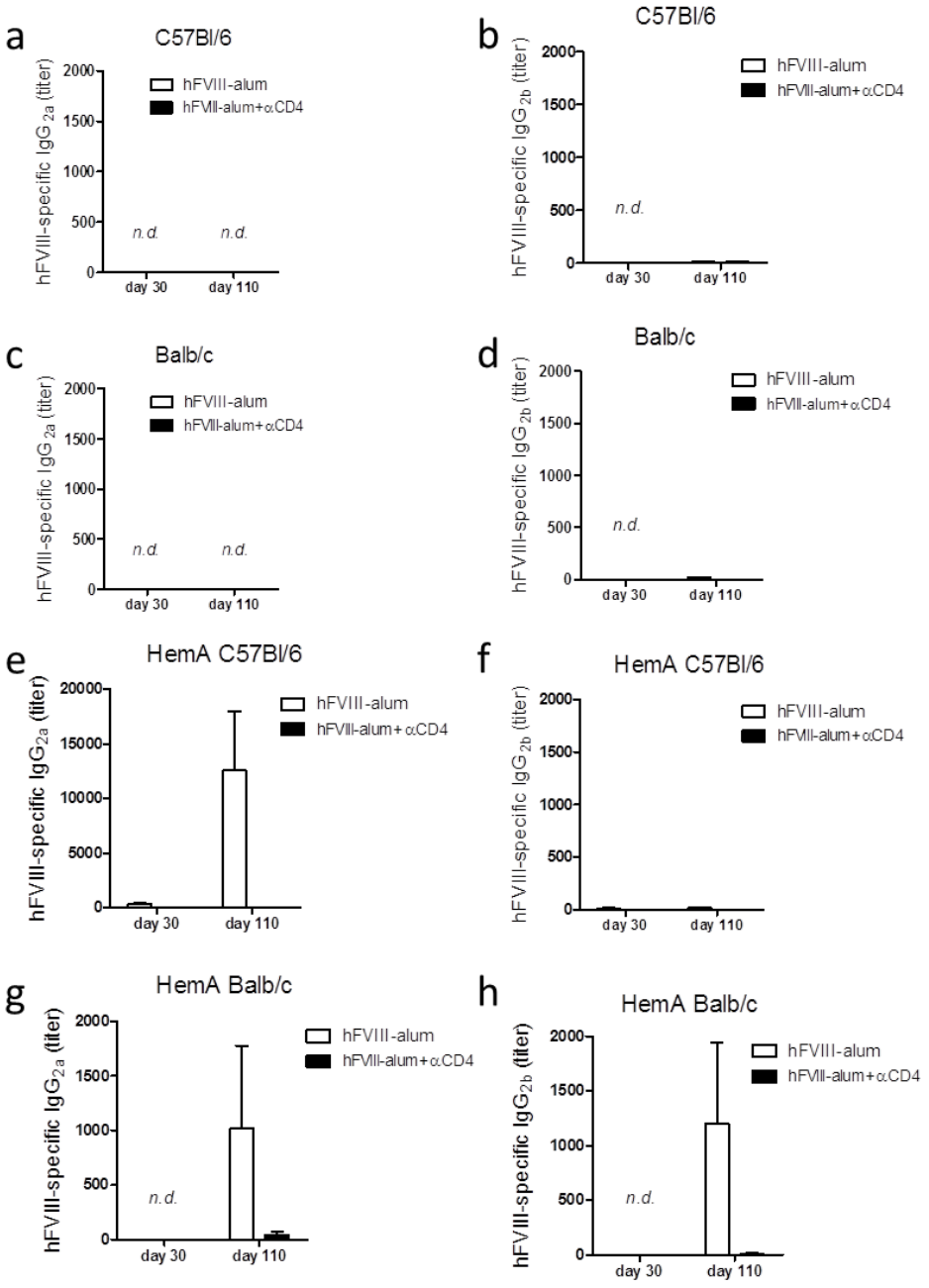
Given the promising pre-clinical results, anti-CD4 MAb was among the very first MAb to enter clinical trials[329, 330]. The outcome of those early clinical trials was disappointing, which led to the search for alternative tolerance-inducing strategies. In retrospect, however, it is possible that the low efficacy was due to immaturity of the field. In fact, clinical trials in patients with rheumatoid arthritis and multiple sclerosis were not performed with humanized MAb, and the low efficacy correlated well with the generation of Ig targeting the therapeutic MAb[331]. In addition, a well known clinical trial in asthma a different anti-CD4 MAb was used (keliximab) that caused significant T cell depletion[323]. More recently, pre-clinical studies with a humanized non-depleting anti-CD4 have shown it could induce tolerance in non-human primates to foreign Ig[217]. It is possible that the use of alum as an adjuvant, as we now describe, will allow tolerance induction to hFVIII but also to other therapeutic recombinant proteins, namely in enzyme replacement therapy.

It is well established that the primed immune system is more resistant to tolerance inducing strategies. Nevertheless, most clinical situations would require tolerance induction in an immunized individual. In hemophilia, FVIII inhibitors are only produced in a proportion of patients. Therefore, tolerance induction should only be initiated in the patients that would require this intervention, and those patients can only be identified after the onset of inhibitors. However, the study of tolerance inducing strategies in mice previously immunized to hFVIII has been difficult due to the long half-life of hFVIII-specific IgG – the experimental readout. As a consequence, most studies in mice are restricted to the ability to induce

tolerance at the time of the initial exposure to hFVIII – an event that is controlled by the clinician. We developed an experimental system to study the effect of hFVIII-alum + anti-CD4 on the primed immune system (Figure 5). Instead of removing pre-existent hFVIII-specific IgG by plasmapheresis – something that we could not do in mice – we transferred primed immune cells to a new host. This way it became possible to confirm that our protocol could prevent *de novo* production of hFVIII-specific antibodies by the primed cells. Such adoptive cell transfer system is unlikely to reproduce in its entirety the characteristics of a primed immune system. However, given the fact that anti-CD4 is able to prevent allergic airways disease in mice pre-sensitized to an allergen (house dust mite), it is likely that following clearance of circulating hFVIII inhibitors treatment with hFVIII-alum + anti-CD4 will prevent subsequent antibody production. Consistent with our data, a report has shown that MAbs targeting ICOS and CD154 can prevent memory plasma cells to respond to FVIII[360].

Taken together our results show that alum, in spite of its common use to boost immune responses, can be effective in enhancing the ability of non-depleting anti-CD4 MAb to induce long-term and antigen-specific tolerance to hFVIII in mice. The tolerogenic mechanism relies on IL-10 and is independent of $Foxp3^+$ Treg cells. We believe that the use of alum, or other adjuvants, may constitute a strategy that could be applied to different immunogenic therapeutic proteins and other tolerance-inducing MAbs. In this case, our findings may be of interest beyond the field of hemophilia, namely in regard to enzyme replacement therapy.

Supplementary Figures



Supplementary Figure 1. *Non-depleting anti-CD4 prevents production of hFVIII-specific IgG2a and IgG2b in HemA mice.* C57Bl/6 HemA (A, C) or BALB/c HemA mice (B, D) were treated with hFVIII-alum and anti-CD4 MAb (or an isotype control) as described in Figure 1A. The serum titres of hFVIII-specific IgG2a (A, B) or IgG2b (C, D) were quantified by ELISA at days 30 and 110 post-initial treatment. (A) The titre of hFVIII-specific IgG2a from C57Bl/6 HemA mice, at day 30 and at day 90, following multiple exposures to hFVIII, was significantly reduced in mice treated with anti-CD4 (n=8; $p<0.001$). (B) In BALB/c HemA mice there was no detectable hFVIII-specific IgG2a at day 30, but at day 90 there was a significant titre in mice treated with the isotype control (n=8; $p<0.001$). (C) Serum hFVIII-specific IgG2b remained low in C57Bl/6 HemA mice throughout the experiment (n=8; non-significant). (D) BALB/c HemA mice displayed a significant increase in the serum titres of hFVIII-specific IgG2b at day 90, that was prevented following treatment with anti-CD4 (n=8; $p<0.001$).

Induction of Foxp3-independent dominant transplantation tolerance - Boosting immune tolerance by activation of complementary regulatory mechanisms

Introduction

Since the pioneering work of Medawar and colleagues several protocols have been proposed to achieve transplantation tolerance in the adult [352, 361-363]. One way to achieve robust tolerance is based on the induction of hematopoietic chimerism following transplantation of donor hematopoietic stem cells (HSC), leading to a recessive state of tolerance independent of $Foxp3^+$ regulatory T (Treg) cells [364]. Other approaches, namely the use of monoclonal antibodies (MAbs) targeting T cell co-receptors (CD3 or CD4) or co-stimulation (CD154), could lead to dominant transplantation tolerance in several animal models [142, 162, 365-368]. Such dominant tolerance was found to be associated with the peripheral induction of Foxp3+ Treg cells [340, 369]. In fact, it has been shown that Foxp3-deficient mice cannot be tolerized with protocols leading to dominant tolerance, such as non-depleting anti-CD4 [370].

Many MAbs that can induce dominant transplantation tolerance were also shown able to induce tolerance in autoimmunity, often associated with the induction of $Foxp3^+$ Treg cells [371, 372], as well as tolerance to allergens or model antigens [373, 374].

We became interested in the mechanisms leading to tolerance induction to antigens, and how adjuvants may influence the process following our observations that it is possible to induce tolerance to a protein with anti-CD4 in a mouse model of allergic airways disease [374], but difficult to induce tolerance to factor VIII (FVIII) with the same MAb (or with anti-CD154) in a mouse model of hemophilia [341, 342]. In fact, when the same antigen (FVIII) is expressed in tissues following gene therapy it can be readily tolerized [343]

We therefore investigated the mechanisms leading to tolerance to a defined antigen (ovalbumin, OVA) when delivered as part of a skin graft (from OVA-actin transgenic mice) or delivered as OVA-alum i.p. We used OVA-specific TCR transgenic mice in two different genetic backgrounds (OT-II and DO11.10) and the same tolerance-inducing method (non-depleting anti-CD4 MAb).

Given the known association of $Foxp3^+$ Treg cells and dominant tolerance, it was surprising to find that unlike tolerance to OVA-expressing skin grafts, the induction of tolerance to OVA-alum under CD4-blockade was Foxp3-independent as it could be induced in Foxp3-deficient mice. In this setting the tolerance induction triggered the reduction of the number of effector T cells, and simultaneously led to a IL-10-dependent tolerance state. It may therefore be possible to take advantage of Foxp3-independent tolerance protocols to boost the efficacy of tolerance-dependent tolerance induction.

Materials and methods

Animals. C57Bl/6, BALB/c, C57BL/6-Tg(ACTB-OVA)916Jen/J (Jackson Laboratories), OT-II.Rag1$^{-/-}$ (Taconic), DO11.10.Rag1$^{-/-}$ (Jackson Laboratories), C57Bl/6.IL-10$^{-/-}$, C57Bl/6.Rag2$^{-/-}$, OT-II, DO11.10 and Wsh/Wsh mice were bred and maintained under specific pathogen-free (SPF) facilities at the Instituto Gulbenkian de Ciência. Experimental animals were sex-matched and between 6 and 8 weeks of age. The T/B monoclonal mice (T-Bmc, 17/9 DO11.10.RAG1$^{-/-}$) bear monoclonal populations of T and B lymphocytes specific for chicken ovalbumin (OVA) 323–339 and HA of influenza virus, respectively [345]. These mice were kept under SPF conditions at the Skirball Institute Central Animal Facility, New York University Medical Center.

Transplantation. Transplantation experiments were conducted by grafting full thickness skin on the lateral flank of recipient mice. Grafts were observed daily and considered rejected when no viable donor skin was present. Procedures were conducted in accordance with guidelines from the Animal Use and Institutional Ethical Committees and approved by Direcção Geral de Veterinaria or New York University School of Medicine Institutional Animal Care and Use Committee (New York).

Sensitization. Animals were sensitized, at the times described in the text, by i.p. injection of 10 μg of ovalbumin (OVA, grade V; Sigma, St Louis, USA) or β-lactoglobulin (Sigma), previously run through a DetoxyGel column (Pierce, Rockford, USA) following manufacturer instructions, and suspended in 2.0 mg of endotoxin-free aluminum hydroxide (Alu-gel-S, Serva, Heidelberg, Germany). Mice were intranasally challenged with 50 μg

of OVA in pyrogen-free saline and sacrificed 24 hours after the last challenge.

Antibodies. Non-depleting anti-CD4 (YTS177) [153] and the isotype control rat anti-dog CD4 (YKIX302) MAbs were produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland), and purified from culture supernatants by 50% ammonium sulfate precipitation, dialyzed against PBS, and the purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Bronchoalveolar Lavage (BAL). The airways were washed through the trachea by slowly infusing and withdrawing 1 ml of cold PBS 10% BSA (Sigma) three times. Eosinophilia was quantified by flow cytometry using GR-1-FITC (eBiosciences, San Diego, CA, USA), CCR3-APC (BD Pharmingen, San Diego, USA), and MHC-class II-Percp.Cy5.5 (eBiosciences, San Diego, CA, USA), with the eosinophils identified based on the SSC/FSC profile and as the GR1intMHCclass II-CCR3- cells [326].

Histology. Lungs were perfused with 4% formalin solution (Sigma), collected and sectioned. Staining was performed using hematoxilin/eosin, and mucus containing cells were revealed using a periodic acid-Schiff (PAS) stain. Photographs were taken using a Leica DM2500 microscope and a Leica DFC420 camera.

Quantification of immunoglobulins and cytokines. Serum titers of OVA-specific IgG1, IgG2a, and IgE were measured by ELISA using the following kits: IgG1 and IgG2a (SouthernBiotech, Birmingham, USA) with anti-OVA IgG1 standard from Serotec, Oxford, UK; IgE (BD-Pharmingen) with anti-OVA IgE standard from Abcam (Cambridge, UK). Cytokine titers were

determined in fresh lung tissue homogenate supernatants. Lung tissue was collected, homogenized in 100 mg/ml in HBSS (Gibco, Carlsbad, USA), centrifuged at 800g for 10 min and the supernatant collected. The ELISA assays were performed according to manufacturer's instructions using the following kits: IL-13(Peprotech, London, UK); IL-4 and IL-5 (BD-Pharmingen).

CFSE staining and adoptive cell transfers. Single cell suspensions from spleen and LN from DO11.10.Rag$^{-/-}$ mice were ressuspended at 5x10^{7} cells/ml and stained with 5 µM of Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Carlsbad, USA). The cells were washed, ressuspended in saline and injected i.v., in the tail vein of BALB/c mice at a total of 3.5x10^{7} cells per animal.

Flow cytometry. Cells were stained for flow cytometric analysis with CD25-AlexaFluor488 (PC61; produced and conjugated in house), CD25-PE-Cy7 (PC61.5; eBioscience), CD3-PE-Cy7 (145-2C11; eBioscience), CD8-APC-AlexaFluor750 (53-6.7;), CD4-PerCp (RM4-5; BD Pharmigen), the DO11.10 TCR-specific KJ1-26 MAb conjugated with PE or APC (BD Pharmigen) and Vβ5.1/5.2 TCR-FITC (MR9-4; eBioscience) plus Vα2 TCR-eFluor 450 (B20.1; eBioscience) were used to determine the TCR transgenic populations in the OT-II mice. Cells were then washed and fixed using Foxp3 Staining Set (eBioscience). The cells were stained for intracellular cytokines and Foxp3 with Foxp3-APC (FJK-16s; eBioscience), IFN-γ-FITC (XMG1.2; eBioscience), IL-13-PE (eBio13A; eBioscience), IL-4-FITC (BVD6-24G2; eBioscience) and IL-5-APC (TRF K5; BD Pharmigen). Apoptotic cells were identified with Annexin V-biotin (BD Pharmigen) and Streptavidin-APC-Cy7 (eBioscience) labeled in Annexin V Binding Buffer (BD Pharmigen) according to manufacturer protocol. Propidium iodide solution was added right before

the cells were analyzed. Four and six color analyses were performed using a LSR Fortessa (BD Bioscience). The analysis gate was set on the forward and side scatters to eliminate cell debris and dead cells.

Statistical analysis. Statistical significance was determined using the two-tailed non-parametric Mann-Whitney test and the log rank method. Statistical analysis was performed using Prism 4.0 (GraphPad, San Diego, USA) and P values <0.05 were deemed significant (*, P<0.05; **, P<0.01; ***, P<0.001).

Results

CD4-blockade can induce immune tolerance to OVA in TCR-transgenic mice.

We have previously shown that long-term antigen-specific tolerance to allergens (house dust mite) can be achieved in mice [374]. The cellular and molecular mechanism underlying the tolerance state remained, however, to be elucidated. In order to follow the fate of antigen-specific cells as tolerance is established we used TCR-transgenic mice. We first investigated in DO11.10 mice whether tolerance to a model antigen (OVA) could still be induced when most of the T cell repertoire is directed towards that antigen. We found that CD4-blockade could prevent allergic airways disease induced with OVA in OVA-specific TCR-transgenic mice (Figure 1). DO11.10 mice treated with non-depleting anti-CD4 were protected from

peri-bronchiolar inflammatory infiltrates and hyperplasia of goblet cells (Figure 1A) as well as airways eosinophilia (Figure 1B). Furthermore the titers of OVA-specific IgG1 and IgE were significantly reduced in anti-CD4-treated mice (Figure 1D, E). We also found that CD4-blockade prevented an increase in the concentration of Th2-type cytokines (IL-4, IL-5, and IL-13) compared to animals treated with an isotype control (Figure 1F). We confirmed that these results are reproducible in a distinct genetic background using OT-II mice, a second strain of OVA-specific TCR-transgenic animals (Supplemental Figure 1). Note that the isotype of the anti-CD4 MAb is non-depleting as we have shown, for instance, by showing that it does not lead to alteration of the number of CD4 T cells *in vivo* that are not engaged in an immune response at the time of antibody treatment [372].

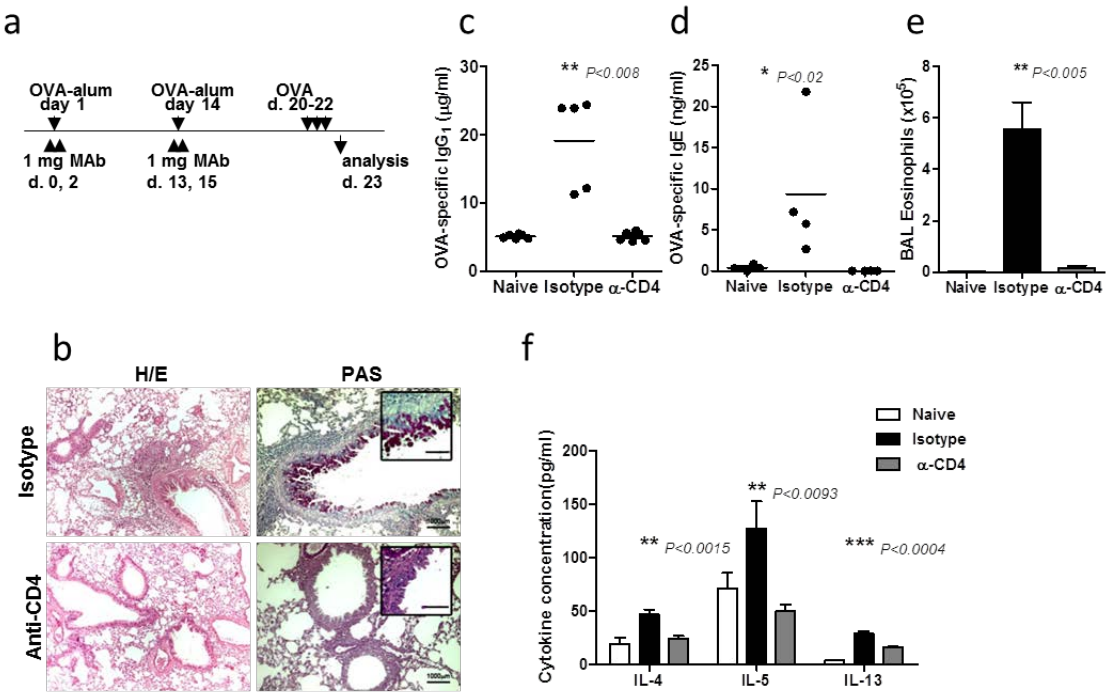


Figure 1. *Tolerance induction with non-depleting anti-CD4 MAb is robust enough to prevent allergic airways disease in TCR transgenic mice*. (A) OVA-specific female DO11.10 mice were sensitized with 20 µg OVA-alum i.p. and challenged with 50 µg OVA in saline i.n. on the indicated days. Some animals were treated with 1 mg anti-CD4 or an isotype control. Naive animals, not subjected to any intervention, were also studied as a control group. (B) Histological sections of lung tissue were stained with haematoxylin/eosin (left) and PAS (right). Airways from anti-CD4 treated mice show a reduction in the inflammatory infiltrate and an absence of goblet cell hyperplasia. Scale bar: 100 µm (25 µm in the inset). (C) Mice treated with anti-CD4 have a reduced number of eosinophils in the BAL (n=6, *P*<0.01). (D) The serum levels of OVA-specific IgG1 and (E) IgE are significantly reduced in anti-CD4 treated mice (n=6, *P*<0.001). (F) The concentration of Th2-type cytokines (IL-4, IL-13 and IL-13) in lung homogenates were markedly diminished in anti-CD4 treated mice (n=6, ***P*<0.01 or ****P*<0.001). (A-F) Data are representative of two independent experiments.

Overall, these results show that CD4-blockade is able to prevent allergic airways disease, even in TCR-transgenic mice where most T cells are specific to the antigen driving the pathology.

Immune tolerance to OVA is Foxp3-independent

Given the high frequency of OVA-specific T cells, and the ability to identify these cells, it was possible to monitor changes in the frequency of OVA-specific T cells with different functional characteristics. Mice treated with anti-CD4 MAb showed a reduced frequency of Th2-type OVA-specific T cells in the mediastinal lymph nodes (MLN) (Figure 2A). In addition, these animals did not show an increased number of Th1 IFN-γ^+ cells (Figure 2A). We also found that the frequency of OVA-specific Foxp3$^+$ Treg cells did not change with the tolerogenic treatment (Figure 2B). Furthermore, DO11.10.Rag$^{-/-}$ mice, devoid of thymic derived Foxp3$^+$ Treg cells [4], subjected to the same tolerance induction protocol did not develop detectable Foxp3$^+$ T cells in the spleen or LNs (Figure 2C).

Previous studies in transplantation have implicated the de novo generation of Foxp3+ Treg cells, in a TGF-β-dependent process, in tolerance induced with anti-CD4 or co-stimulation blockade [340, 369]. Furthermore, mast cells were shown to be critical for the establishment of such transplantation tolerance [375].

We used mast cell deficient Wsh/Wsh mice, with a mutant allele at the W (c-kit) locus, to investigate whether mast cells were required for tolerance induction [376, 377]. The sensitization protocol was slightly modified in order to maximize airways inflammation in the C57Bl/6 genetic

background, with three injections of 10 µg OVA-alum on days 1, 7, and 14. The animals received three i.p. injections of MAb on days 0, 2, and 14. We observed that mast cell deficient mice treated with anti-CD4 are protected from airways allergic inflammation to the same extent as littermates with normal numbers of mast cells (Figure 2D).

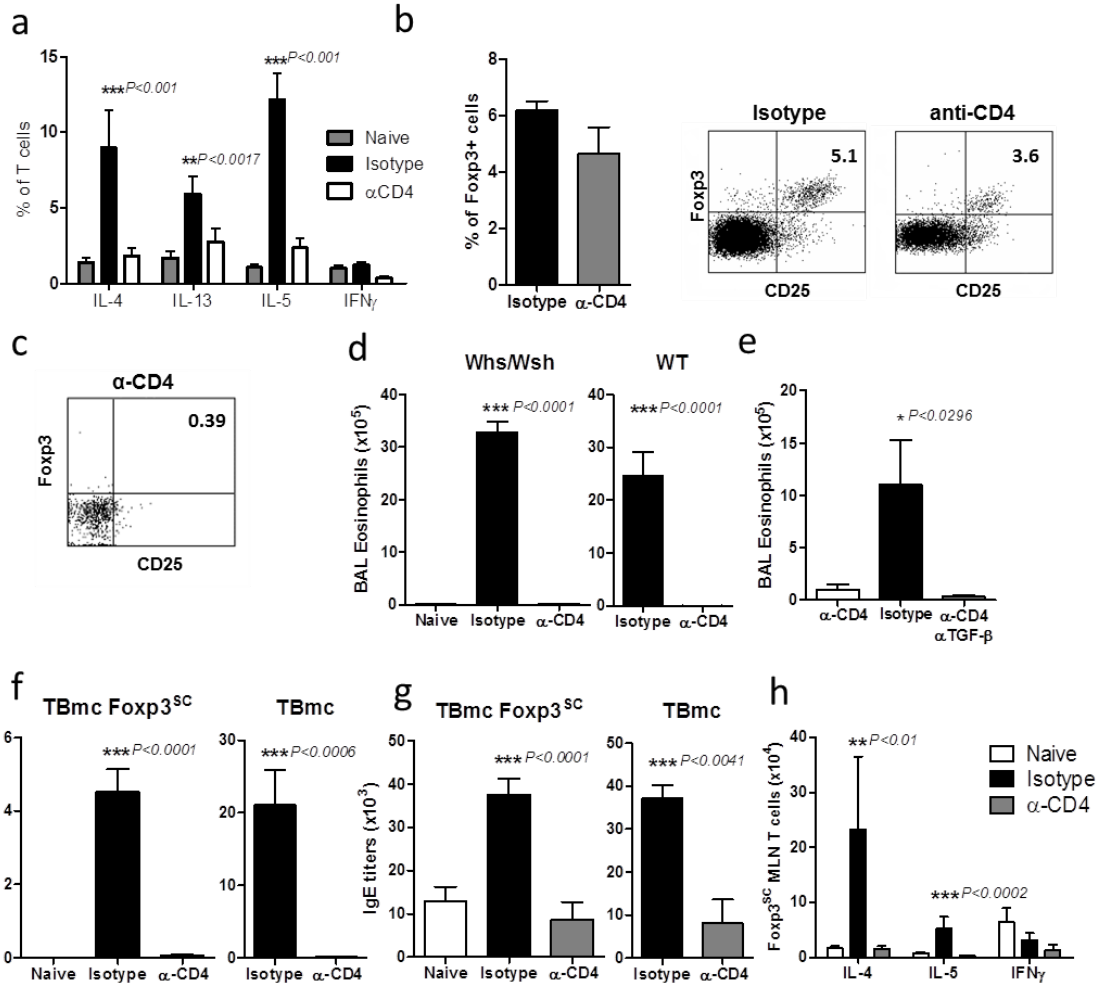


Figure 2. *CD4-blockade leads to reduction of Th2 cells without concomitant Treg expansion*. (A) OVA-specific T cells in MLN containing IL-4, IL-5, and IL-13 from animals treated with anti-CD4 MAb were reduced to numbers similar to mice not

exposed to OVA (n=6, ***P*<0.01), while the number of IFN-γ^{+} OVA-specific T cells did not increase. (B) The frequency of OVA-specific Foxp3^{+} Treg cells remained unchanged in anti-CD4 treated mice. (C) There is no induction of Foxp3^{+} cells in DO11.10 Rag$^{-/-}$ animals treated with anti-CD4 MAb (D) Tolerance to OVA can be induced in mast cell deficient mice. We quantified BAL eosinophils from mast cell-deficient Wsh/Wsh mice and wild-type (WT) littermates sensitized in the presence of anti-CD4 or an isotype control (as described in Figure 1A). Protection from airways eosinophilia following CD4-blockade was similar in both groups of mice (n=4, *** *P*<0.001). (E) BALB/c mice, treated as described in Figure 1A, were also injected with 1 mg of a neutralizing anti-TGF-β MAb on days -1, 2, 7, 13, 15. Neutralizing MAbs did not abrogate the protective effect of CD4-blockade (n=6, **P*<0.05). (F) Eosinophils in the BAL of T-Bmc Foxp3sf mice and T-Bmc Foxp3-sufficient littermates sensitized with OVA-HA-alum and challenged with OVA-HA i.n. according to the protocol described in Figure 1A. CD4-blockade prevented BAL eosinophilia in Foxp3-deficient mice, as well as in Foxp3 sufficient controls (n=8, *P*<0.001). (G) We also confirmed that Foxp3 deficient mice treated with anti-CD4 had low levels of serum IgE (n=8, *P*<0.001). (H) Furthermore, the antigen-specific T cells (TCR transgenic) from Foxp3-deficient mice exposed to CD4-blockade did not polarize towards an IL-4, IL-5 or IFN-γ producing phenotype (n=8, *P*<0.001). (A-H) Data are representative of two independent experiments.

We also neutralized TGF-β (using the MAb 1D11) in BALB/c mice during tolerance induction. This neutralization did not hamper protection from allergic airways disease (Figure 2E).

In order to definitely exclude a contribution for Foxp3^{+} Treg cells from tolerance induction with anti-CD4 we used T-Bmc.Rag$^{-/-}$ mice deficient in Foxp3 (T-Bmc Foxp3^{sf}) [326]. These mice bear OVA-specific TCR transgenic T cells and HA-specific BCR knock-in B cells. They do not develop an autoimmune condition in the absence of Foxp3 since all their lymphocytes are monoclonal and non-self reactive. These mice were sensitized with OVA-HA-alum i.p., and latter exposed to OVA-HA i.n. Foxp3-deficient animals treated with anti-CD4 displayed similar protection from allergic airways disease as Foxp3 sufficient littermates (Figure 2F). The Foxp3

deficient animals were also prevented from generating IgE and did not display an increase in Th2 cells in MedLN (Figure 2G, H).

Tolerance induction to OVA-expressing skin grafts in TCR-transgenic mice with CD4-blockade is Foxp3-dependent

Given that our data on tolerance induction is independent of Foxp3 in OVA-specific TCR transgenic mice, is in apparent contradiction with previous reports showing transplantation tolerance in TCR-transgenic mice is Foxp3 dependent [340, 369], we assessed whether in OVA-specific TCR-transgenic mice tolerance could be induced to OVA-expressing skin grafts. We transplanted OT-II.Rag$^{-/-}$ mice with skin from OVA-actin transgenic mice. The animals were treated with 1 mg anti-CD4 (or isotype control) on day 0 and 2 after transplantation. We found CD4-blockade induced long-term skin graft survival (Figure 3A), with these mice showing a population of induced Foxp3^{+} Treg cells in draining LN and spleen (Figure 3B). To prove the Foxp3-dependency of transplantation tolerance we repeated the experiment with Foxp3-deficient OT-II.Rag$^{-/-}$.Foxp3^{sc} mice. We found that animals lacking functional Foxp3 could not be tolerized and readily rejected the skin grafts (Figure 3C).

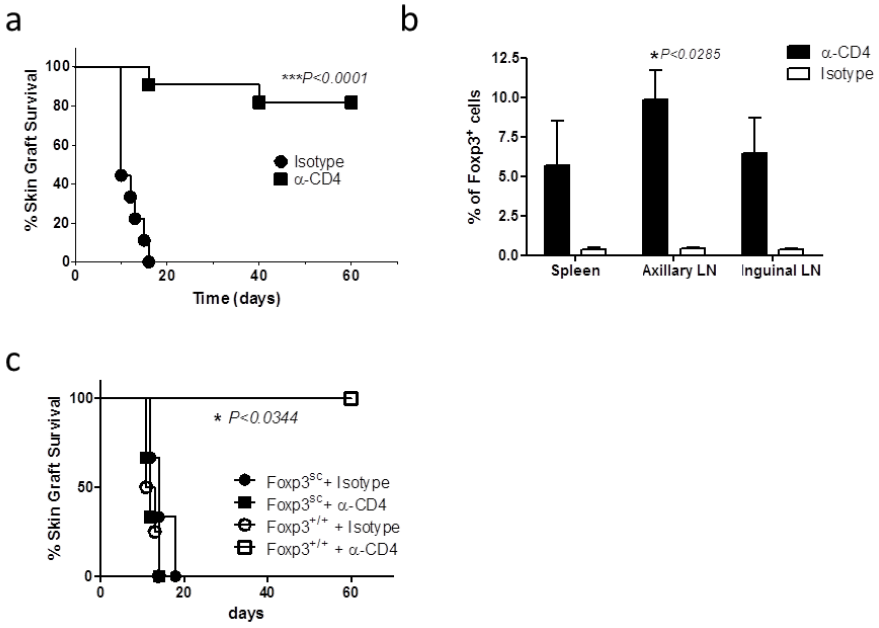


Figure 3. *Acceptance of OVA-expressing skin grafts by TCR-transgenic mice following CD4-blockade is Foxp3-dependent*. (A) OT-II.Rag$^{-/-}$ mice were transplanted with skin grafts from OVA-actin transgenic mice in the presence of anti-CD4 MAb or an isotype control. Treatment with anti-CD4 led to long term acceptance of skin grafts in most animals (■, n=6, median survival time (MST)>100 versus ● MST=10d in the isotype control group; ***P<0.001). (B) We observed the emergence of Foxp3^{+} Treg cells in the animals tolerized to OVA-expressing skin grafts (n=6, P<0.05). (C) The same experimental protocol in Foxp3-deficient OT-II.Rag$^{-/-}$.Scurfy mice failed to prolong graft survival (■, n=4, MST=13d). Transplant rejection occurred at the same time as control OT-II.Rag$^{-/-}$ (○, n=4, MST=12d) or OT-II.Rag$^{-/-}$.Scurfy mice (●, n=4, MST=13d) treated with an isotype control. (A-C) Data are representative of two independent experiments.

Tolerance induction with OVA-alum + anti-CD4 has a recessive and dominant component

To investigate the impact of anti-CD4 treatment in T cell proliferation and survival, CFSE-labeled OVA-specific T cells from DO11.10.Rag$^{-/-}$ mice

were adoptively transferred into BALB/c mice. The host animals were sensitized with OVA-alum i.p. in the presence or absence of anti-CD4 treatment. Lung, mediastinal LN (MLN), mesenteric LN, and spleen T cells were isolated at day 4 and 7 following treatment and analyzed by flow cytometry.

Consistent with a recent report on T cell response to OVA-alum administered i.p. [378], MLN displayed the highest number of T cells responding to the antigen. An observation of the CFSE dilution profile in OVA-specific T cells isolated from MLN may suggest that anti-CD4 treatment prevents T cell proliferation, as the undivided cells are a greater proportion of the TCR-transgenic population unlike in mice injected with OVA alone (Figure 4A). But if anti-CD4 would exclusively target T cell proliferation one would expect the absolute number of TCR transgenic cells recovered from the undivided CFSE gate to be similar in mice treated with anti-CD4 and animals not exposed to OVA where cells do not divide at all. The enumeration of undivided and divided TCR transgenic cells from the different experimental groups does not support the hypothesis of inhibition of proliferation (Figure 4B). In fact, the number of undivided cells from anti-CD4 treated animals was remarkably similar to animals treated with OVA alone, where extensive proliferation does occur, and significantly lower than animals not treated with OVA. The difference between OVA and OVA + anti-CD4 treated groups is not in the undivided T cells but rather on the accumulation of T cells that underwent cell divisions. Given these data, either the anti-CD4 treatment is targeting the deletion of undivided cells or inducing the cell death of T cells entering cell cycle.

The analysis of the TCR transgenic cells positive for the apoptosis marker annexin V showed that the frequency of apoptotic cells was similar

between undivided cells from all experimental groups (Figure 4C). Only the cells undergoing cell divisions from anti-CD4 treated mice had a significant increase in the frequency of apoptotic cells. As a consequence, besides a possible effect on T cell proliferation, anti-CD4 treatment preferentially induces the apoptosis of activated cells.

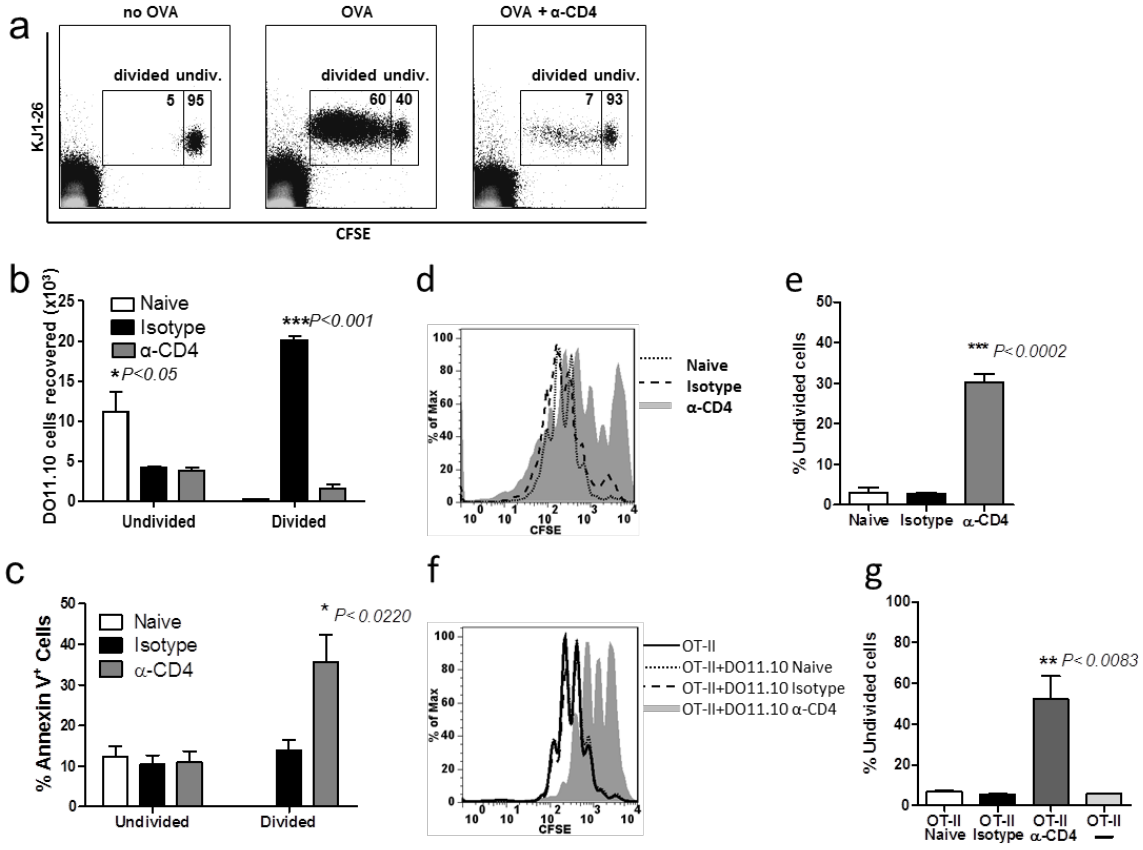


Figure 4. *Tolerance induction with anti-CD4 leads to reduction of effector T cells and emergence of an anergic and regulatory T cell population*. Splenic and LN T cells from DO11.10.Rag$^{-/-}$ mice were stained with CFSE and injected i.v. into sex-matched BALB/c hosts. Recipient mice were then treated with OVA-alum i.p. in presence or absence of anti-CD4. Control animals were not exposed to OVA. (A) Analysis of MLN at day 4. Dot plots were gated on CD3^{+} T cells. The numbers represent the frequency of T cells within the TCR transgenic cells. (B) Absolute number of DO11.10.Rag$^{-/-}$ cells recovered from MLN contained in each of the CFSE

regions displayed in (A). The difference in cell frequency between undivided cells from mice not injected with OVA is significantly higher than T cells from mice treated with OVA or OVA+anti-CD4 (n=5, **P*<0.05). (C) Frequency of apoptotic cells (labelled with annexin V) within cells that did not divide or cells that have divided. The frequency of apoptotic cells is significantly higher within the T cells undergoing cell divisions from anti-CD4 treated animals (n=5, *P*<0.05). (D, E) *In vitro* proliferation of CFSE labelled OVA-specific T cells from DO11.10.Rag$^{-/-}$ mice (naive, or sensitized with OVA with or without anti-CD4) following three day culture in presence of DCs from BALB/c mice and $OVA_{323-339}$ peptide. OVA-specific T cells from mice tolerized with anti-CD4 show impaired proliferative ability (n=6, ****P*<0.001). (F, G) Proliferation of CFSE-labelled T cells from naive OT-II.Rag$^{-/-}$ mice co-cultured for three days with T cells sorted from naïve, isotype, or anti-CD4 treated DO11.10.Rag$^{-/-}$ mice. Stimulation was provided by DCs from (BALB/c x C57Bl/6) F1 mice loaded with $OVA_{323-339}$ peptide. The OVA-specific T cells sorted from mice tolerized to OVA, but not from other mice, were able to prevent proliferation of effector OT-II cells (n=6, ***P*<0.01).

However, some antigen-specific cells can still be isolated from tolerant mice, particularly when tolerance is induced in TCR-transgenic mice. We therefore investigated the *in vitro* properties of OVA-specific T cells from DO11.10.Rag$^{-/-}$ mice tolerized with the protocol described. By labeling the cells with CFSE and incubating those with OVA loaded bone marrow DCs; we could compare the proliferative ability of the OVA-specific T cells isolated from naive mice, mice sensitized with OVA-alum, or animals treated with anti-CD4 at the time of OVA-alum sensitization. We found that cells from tolerized mice proliferated less than T cells from the other two groups with a significant arrest in a non proliferative state (Figure 4D, E).

To investigate whether these cells are suppressive, we labeled OVA-specific T cells from naive OT-II mice with CFSE, and incubated these responder cells with OVA-specific cells from DO11.10.Rag$^{-/-}$ mice (naive, sensitized with OVA-alum, or tolerized with OVA-alum + anti-CD4). The

antigen presenting cells were OVA-loaded DCs from (BALB/c x C57Bl/6)F_1 mice, able to present OVA-peptides both in the context of H-2^b and H-2^d (therefore excluding a possible competition effect of both T cell populations for the same MHC molecules). We found that T cells from tolerant mice moderately suppress the proliferation of responder OT-II cells (Figure 4F, G).

Taken together, these data show that CD4-blockade induces a significant reduction of effector T cell numbers by apoptosis and, simultaneously, the surviving T cells display moderate anergic and suppressive properties.

IL-10 is critical for tolerance induction

We then investigated whether the dominant component of the tolerance state was IL-10 dependent. For that we compared tolerance induction to OVA-expressing skin grafts or to OVA-alum in WT and IL-10-deficient hosts.

In transplantation experiments we found that transplantation tolerance could be readily achieved in WT mice but not in IL-10-/- mice treated with anti-CD4 (Figure 5A).

Similarly, when IL-$10^{-/-}$ mice were sensitized with OVA-alum i.p. in presence of anti-CD4 they did not become protected from allergic airways disease as it was manifest by airways eosinophilia (Figure 5B).

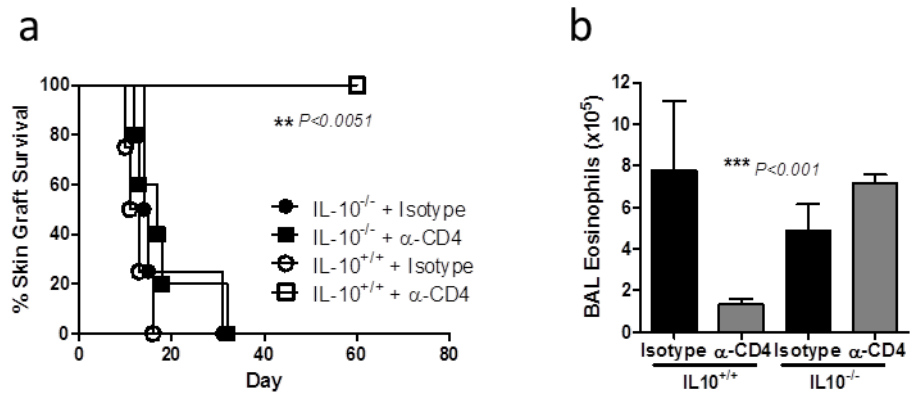


Figure 5. *Induction of immune tolerance with anti-CD4 is IL-10-dependent*. (A) IL-$10^{-/-}$ mice and WT controls were transplanted with skin grafts from OVA-actin transgenic mice in the presence of anti-CD4 MAb. The grafts were rejected in anti-CD4-treated IL-$10^{-/-}$ mice (■, n=5, MST=13d) at the same time as in untreated WT (□, n=6, MST=12d) or untreated IL-$10^{-/-}$ mice (●, n=5, MST=13d). (B) Allergic airways disease was induced in IL-$10^{-/-}$ mice and WT controls, some animals were treated with non-depleting anti-CD4 as described in Figure 1A. We used BAL eosinophilia as a surrogate marker of allergic airways disease. We found that CD4-blockade could prevent airways eosinophilia in WT but not in IL-$10^{-/-}$ mice (n=6, ***P<0.001).

Tolerance induction to OVA-alum can lead to OVA-expressing skin graft acceptance

Given the different requirement of Foxp3 expression when tolerance was induced to an OVA-expressing skin graft or to OVA-alum administered i.p. we investigated whether transplant acceptance or protection from allergic airways disease required a specific type of tolerance induction. First, we induced tolerance to OVA-expressing skin graft in C57Bl/6 mice, as described above. Then, at day 30 transplanted mice treated with anti-CD4 (all with surviving skin grafts) or treated with an isotype control (all

rejected) were sensitized with OVA-alum i.p. and subsequently challenged with OVA i.n. as represented in Figure 6A. The challenge with OVA-alum did not lead to graft rejection and the tolerant mice were protected from airways eosinophilia to the same extent as mice treated with anti-CD4 at the time of OVA-alum sensitization (Figure 6C).

We then performed the converse experiment: C57Bl/6 mice were tolerized to OVA-alum or the unrelated antigen β-lactoglobulin (β-Lg)-alum by concomitant treatment with anti-CD4 MAb (Figure 6D). At day 30 following the last administration of the antigen all mice were transplanted with OVA-expressing skin grafts. We found that mice tolerized to OVA-alum i.p. accepted the OVA expressing skin grafts. On the contrary, mice treated with anti-CD4 in presence of an unrelated antigen (β-Lg) readily rejected the OVA-expressing skin grafts.

These data show that the tolerance state is antigen specific (as mice tolerized β-Lg do not support survival of grafts bearing a different antigen), and also show that tolerant mice remain competent to mount immune responses to unrelated antigens.

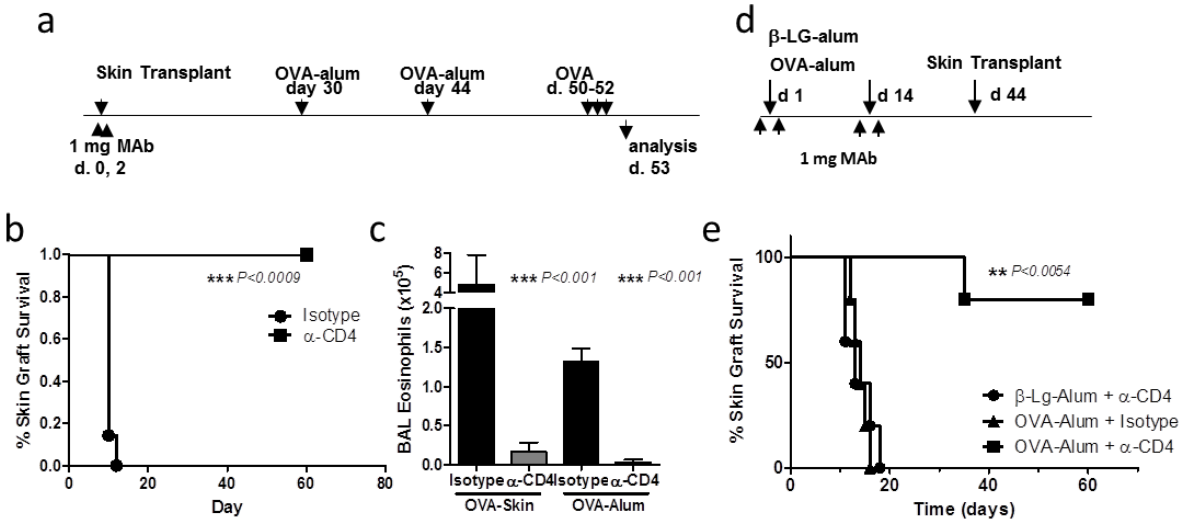


Figure 6. *Tolerance induction to OVA-alum can lead to subsequent skin graft acceptance*. (A) C57Bl/6 mice received skin grafts from OVA-actin transgenic mice in the presence of anti-CD4 MAb. On day 30 after transplantation the mice were sensitized with OVA-alum i.p. and challenged with OVA i.n. according to the scheme. (B) Mice tolerant to OVA-expressing skin grafts did not reject the transplants following sensitization with OVA-alum at day 30 and 44 (n=6, MST=12d vs. MST>60d, ***P<0.001). (C) Mice tolerant to an OVA-expressing skin graft were resistant to the induction of allergic airways disease with the same antigen, to the same extent as mice treated with anti-CD4 at the time of OVA-alum exposure. On the contrary, those animals that had rejected the skin graft had more exacerbated BAL eosinophilia (n=6, MST=12d, ***P<0.001). (D) C57Bl/6 mice were tolerized to OVA-alum or β-LG-alum by concomitant administration of anti-CD4. On the day 30 following last MAb infusion these animals were transplanted with skin grafts from OVA-actin transgenic mice. (E) Tolerance induction with OVA-alum and anti-CD4 30 days prior to transplantation was sufficient for long-term graft acceptance in the absence of additional treatment (■, n=6, MST>60, P<0.01). However, mice tolerized to an unrelated third-party antigen (β-LG) rejected their grafts at the same time as animals that did not receive anti-CD4 (● and ▲, n=6, MST=14d).

Discussion

Our data show that the same tolerance-inducing agent (non-depleting anti-CD4 MAb) can trigger different mechanisms leading to dominant tolerance depending on the context by which the antigen is presented to the immune system. Using TCR transgenic mice it was possible to demonstrate that tolerance to an antigen in a skin graft requires the peripheral induction of $Foxp3^+$ Treg cells, while the delivery of the same antigen through the i.p. route with an adjuvant (alum) triggers a Foxp3-independent tolerance mechanism that relies on IL-10 and apoptosis of effector cells.

Our data demonstrates the critical role of the context in which an antigen is perceived by the immune system for therapeutic induction of tolerance. In fact, it has been paradoxical that the same reagents (such as non-depleting anti-CD4 or anti-154) can readily induce transplantation tolerance to multiple alloantigens [162, 366], including major antigens [162, 366], fail to achieve long-term tolerance to single proteins in the same mouse strains [341, 342]. However, the same antigen (FVIII) that is not tolerized when administered i.v. in the absence of adjuvants [341, 342], appears to be easier to tolerize when expressed in tissues following transduction with viral vectors [343]. Our previous findings showing that robust dominant tolerance can be induced to allergens (house dust mite) in alum [374] raised the hypothesis that it is necessary some adjuvanticity to achieve tolerance with MAbs that target T cell co-receptors or co-stimulation. Such adjuvanticity can be provided by tissue inflammation (in transplantation or viral transduction) or with the use of an adjuvant like alum.

When we investigated the mechanisms leading to immune tolerance induced with OVA-alum administration under CD4-blockade it came as a surprise that the tolerance state was Foxp3-independent. In fact, most studies using anti-CD4 (or MAbs targeting other T cell molecules) have shown that tolerance was a consequence of peripheral $Foxp3^+$ Treg induction and could not be induced in the absence of functional Foxp3 [340, 370]. Importantly, the tolerance state achieved is long term, as mice tolerized with OVA-alum + anti-CD4 accept indefinitely OVA-expressing skin grafts at a later time as shown in Figure 6. In addition the tolerance state is antigen specific and not due to persistence of the tolerance-inducing MAbs, because mice tolerized with β-LG-alum + anti-CD4 reject OVA-expressing skin grafts. These data also demonstrate that tolerant animals remain immunecompetent to mount immune responses towards unrelated antigens.

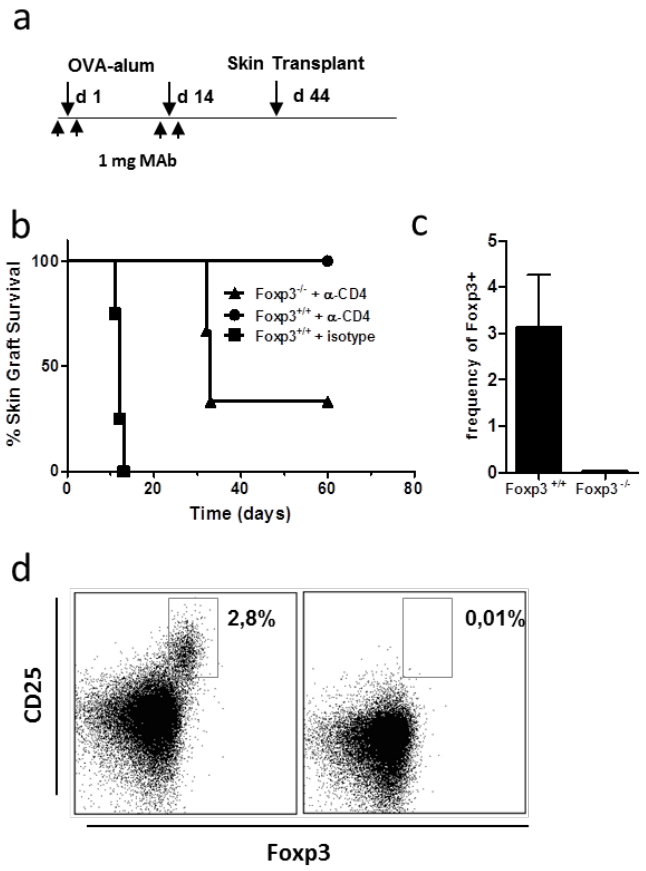
By performing adoptive cell transfers of OVA-specific T cells into WT mice, we could follow the fate of antigen-specific cells as tolerance is induced. We found that CD4-blockade at the time of exposure to the antigen as an effect not only restricted to inhibition of proliferation but also favors apoptosis of the cells as they enter cell cycle. We have recently investigated the impact CD4-blockade, *in vitro*, at the time of activation where we found that the impact on the rate of cell proliferation and apoptosis is small, but with the assistance of mathematical models it was possible to demonstrate that such small effects over time lead to a major difference in the final outcome (Caridade et al, submitted). Previous studies with co-stimulation blockade had also shown that activation under those tolerogenic conditions lead to apoptosis of effector cells [280, 364, 372], but in that case together with the induction of Treg cells [159, 343].

We now found that the impact of apoptosis on the antigen specific T cells is not complete, with several cells surviving deletion. When we assessed those surviving antigen-specific cells we found that they have an anergic and immunosuppressive behavior in spite of lack of Foxp3 expression.

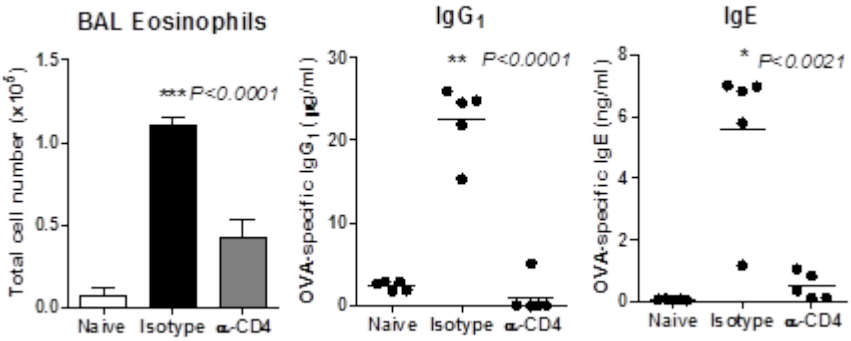
We found that IL-10, unlike Foxp3, is key for the induction of immune tolerance. The implication of IL-10 in transplantation tolerance has been controversial with several studies able to demonstrate long-term transplantation tolerance in the absence of IL-10 [158, 340, 379]. The majority of studies that have shown a strong IL-10 requirement for transplantation tolerance have used donor blood transfusion as part of the tolerance inducing regimen [351, 353, 380]. It is possible that the systemic delivery of antigens mimics the boosting of an IL-10 dependent mechanism (in addition to the recessive mechanisms associated to apoptosis and inhibition of proliferation) that we described for OVA-alum. However, we found that in transplantation tolerance of OVA-expressing skin grafts IL-10, in addition to Foxp3, is essential even in the absence of donor blood transfusion.

Given the fact that dominant transplantation tolerance can be extended to additional antigens present in a new transplanted tissue by a mechanism that was termed *linked suppression* [343], it may be possible to use immune dominant alloantigens to boost tolerance to tissues through a Foxp3-independent mechanism. But more important is the realization that effective tolerance to proteins, such as in allergic diseases or protein replacement therapies (namely in hemophilia or lysossomal storage diseases), may involve robust tolerance mechanisms that are independent of Foxp3.

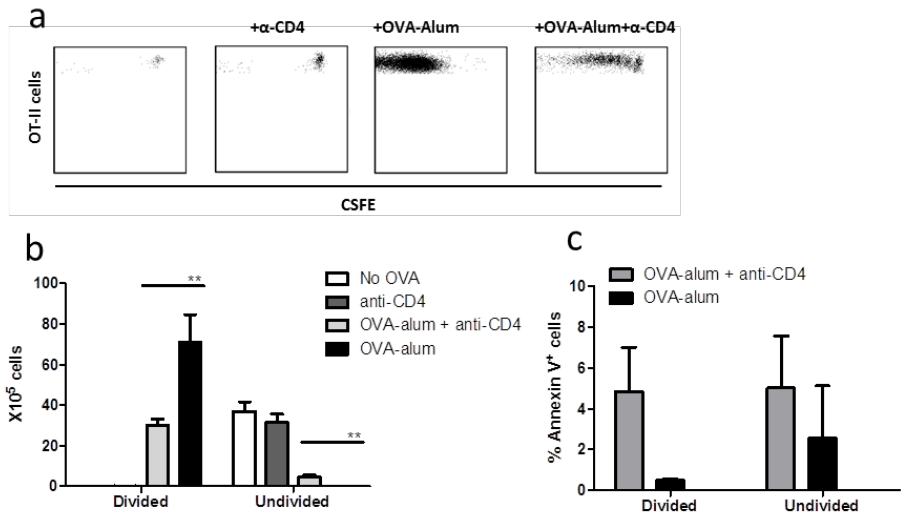
Supplementary Figures



Supplementary Figure 1. *Tolerance induction with OVA-alum can improve subsequent skin graft acceptance in Foxp3 deficient mice*. (A) OT-II Rag$^{-/-}$ Foxp3 sufficient and insufficient mice were trated with OVA-alum in the presence of anti-CD4. On day 30 after treatment, mice received skin grafts from OVA-actin transgenic mice in the presence of anti-CD4 MAb according to the scheme. (B) Foxp3 sufficient mice treated with OVA-alum were tolerant to OVA-expressing skin grafts and did not reject the transplants (n=6, MST=12d vs. MST>60d, ***P<0.001) Foxp3 insufficient mice showed a delayed rejection with some grafts surviving long term (n=3, MST=32). (C) Foxp3 sufficient mice induced low frequency of Foxp3^{+} T cells (n=6, MST=12d, ***P<0.001). (D) Illustrative dot plots of CD25^{+}Foxp3^{+} T cells within the OT-II T cell gate.



Supplementary Figure 2. *Tolerance induction with non-depleting anti-CD4 MAb is robust enough to prevent allergic airways disease in TCR transgenic mice*. (A) OVA-specific female OT-II were sensitized with 20 µg OVA-alum i.p. and challenged with 50 µg OVA in saline i.n. as in Figure 1. Some animals were treated with 1 mg anti-CD4 or an isotype control. Naive animals, not subjected to any intervention, were also studied as a control group. Mice treated with anti-CD4 have a reduced number of eosinophils in the BAL (n=6, $P<0.01$). The serum levels of OVA-specific IgG1 and (E) IgE are significantly reduced in anti-CD4 treated mice (n=6, $P<0.001$, $^{**}P<0.01$ or $^{***}P<0.001$). Data are representative of two independent experiments.



Supplementary Figure 3. *Tolerance induction with anti-CD4 leads to reduction of effector T cells and emergence of an anergic and regulatory T cell population*. Splenic and LN T cells from OT-II.Rag$^{-/-}$ mice were stained with CFSE and injected i.v. into sex-matched BALB/c hosts. Recipient mice were then treated with OVA-alum i.p. in presence or absence of anti-CD4. Control animals were not exposed to OVA. (A) Analysis of MLN at day 4. Dot plots were gated on CD3^{+} T cells. The numbers represent the frequency of T cells within the TCR transgenic cells. (B) Absolute number of OT-II.Rag$^{-/-}$ cells recovered from MLN contained in each of the CFSE regions displayed in (A). The difference in cell frequency between undivided cells from mice not injected with OVA is significantly higher than T cells from mice treated with OVA or OVA+anti-CD4 (n=5, **P*<0.05). (C) Frequency of apoptotic cells (labelled with annexin V) within cells that did not divide or cells that have divided. The frequency of apoptotic cells is significantly higher within the T cells undergoing cell divisions from anti-CD4 treated animals (n=5, *P*<0.05).

Discussion

The use of monoclonal antibodies targeting specific molecules has become an emerging therapy for immune mediated pathologies. Monoclonal antibodies have been used to neutralize inflammatory mediators [381], to deplete cells [382], to skew the immune responses [196], namely by reprogramming the immune system towards tolerance [383]. Antibodies targeting the immune synapse have been described to induce putative Tregs that can suppress effector responses [34, 159] thus tipping the balance towards a tolerant state of the immune system.

This thesis focuses on CD4 blockade, which has been shown to be very effective in preventing transplant rejection and autoimmunity [357, 384] through the induction of a regulatory specific population of $Foxp3^+$ T cells [148, 159]. In addition the tolerogenic effect of CD4 blockade molecule was shown effective in preventing immune responses to soluble proteins, therefore having a beneficial effect on pathogenic Th2 responses, namely in allergic diseases. The mechanism associated with type of tolerance induction, however, seems to be distinct from the tolerant state induced in the context of autoimmunity or transplantation.

Airways inflammation induced by HDM or OVA in mice allowed us to show that CD4 blockade is effective in inhibiting airways hyperreactivity and remodeling, as well as in abrogating Th2 inflammation. Moreover, and for the first time, we were able to show that anti-CD4 treatment, in this animal model of allergic pathology, was not only exerting an anti-inflammatory role but also, and most importantly, creating a state of antigen-specific tolerance. As a consequence, subsequent sensitization and challenges with the same antigen do not result in inflammation, while the immune system remained competent to respond to third-party antigens. A plethora of studies where co-stimulatory and co-activator blocking agents

were used did not distinguish the impact of the therapeutic agent on the onset of inflammation from its potential tolerogenic role [178, 179, 193, 196]. The antigen-specific nature of therapeutically induced tolerance is not easy to demonstrate without crisscross type assays, given potential differences in the "immunogenicity" of different antigens. Therefore, it is necessary to show that the immune system tolerized to a given X protein can respond to a different Y one, but conversely it is possible to induce tolerance to Y, with the tolerant immune system capable to respond to X. This demonstration is of paramount importance to distinguish a state of immune tolerance from non-specific immune suppression. In the present studies we have shown that CD4 T helper cell responses remained unaffected, while other studies have previously shown that CD4 blockade does not impact on the CD8 T cell compartment [385]. We took particular care to separate in time the tolerization and sensitization stages as to avoid any residual presence of the anti-CD4 MAb. Another crucial finding was that primed cells are still susceptible to the tolerizing effect of anti-CD4. Previous studies have shown that non-depleting anti-CD4 preferentially eliminated naive $CD4^+$ T cells rather than memory and effector $CD4^+$ T cells [386]. Furthermore, induction of transplantation tolerance in primed mice required the combined action of anti-CD4 and anti-CD8 MAbs [155]. It could be argued that while in transplantation CD8 T cells participate as effector cells [387], in a Th2-mediated pathology (such as allergic asthma) CD8 T cells do not represent a significant obstacle for tolerance induction [388], thus making the tolerogenic role of CD4-blockade sufficient even in pre-sensitized mice. Possibly, the recently described Tc9 cells, CD8 T cells expressing IL-9, that appear able to contribute to the severity of Th2-dependent airway inflammation [389], do not represent a major obstacle for tolerance induction through CD4 blockade. In addition it is likely that

allergens induce lower frequencies of effector T cells than alloantigens, thus making an immune system primed following transplantation harder to tolerize than when priming is achieved with a model antigen (such as OVA or FVIII) or an allergen (such as HDM).

It was possible to observe that eosinophilia and airway hyperreactivity in anti-CD4-treated previously sensitized mice was reduced. It is hard to quantify the effects of CD4-blockade in the de novo production of immunoglobulins given the pre-existent high titers of IgE and IgG1 induced at the time of initial sensitization. This could be seen as an opportunity to further study the possible beneficial effects of anti-CD4 in chronic asthma models.

Further studying the advantage of CD4 blockade in tolerance to soluble proteins we demonstrated that in a mouse model of hemophilia it is possible to induce tolerance to the coagulation factor used in the therapy of this disease. Again, the induced tolerance was antigen specific and primed cells remained susceptible to regulation. Trying to take one step further, we devised a method of having primed cells without the presence of immunoglobulins masking the permissive state and it was then possible to confirm that anti-CD4 can have an impact even in pre-sensitized cells.

Cardinally our data points to an enhancing effect of the adjuvant alum in the ability of anti-CD4 to induce tolerance. Adjuvants are mainly known for their immunogenic role [390], thus seeming unwise to have such a compound synergizing towards creating tolerance. Alum in particular has many features that could explain its co-action with anti-CD4: it is known to increase the half-life of antigens; it is known its inability to augment cell-

mediated immune responses, especially cytotoxic T-cell responses [391]; and it can modify antigen presentation through inflammasomes [392]; among other roles that have not yet been characterized. We addressed several of these characteristics of alum.

We increased the persistence of hFVIII through the administration of several shots of the protein during the tolerance induction period. We found that the presence of the adjuvant proved to be crucial for long term tolerance and could not be emulated by the simple prolongation of the presence of the antigen. Albeit alum certainly increases the antigen half-life in vivo, this supposed depot-effect has been recently questioned, as it has been shown that the removal of alum deposits following immunization has no effect in reducing the humoral response [393]. We are still addressing any possible role for inflammasomes, using caspase-1- and NALP-3-deficientmice. Preliminary data obtained in our laboratory suggest that neither NALP-3 nor caspase-1 integrity are required for the facilitation of tolerance induction following administration of alum (data not shown). Alum, however, causes an accelerated influx of neutrophils and $GR1^+$ $IL4^+$ eosinophils [393-395] at the site of injection which can readily skew CD4 T cells towards a Th2 response and prime B cells. Instead of direct effects on the lymphocytes themselves, adjuvants seem to enhance T and B cell responses by directly engaging components of the innate immune system [396-398]. As such, different adjuvants can influence the type of effector CD4 T cells response, with some adjuvants promoting Th1 responses while others favor a Th2 polarization [399] and therefore influencing the isotype of antibodies produced by the B cells [400]. Naturally this notion implies that in vaccines the choice of the right adjuvant can represent an advantage regarding the nature of the protective memory cells generated.

But it could also mean that the type of adjuvant could help directing the type of tolerance mechanisms recruited at the time tolerance is induced with anti-CD4. Therefore, for Th2-mediated diseases the use of alum is beneficial, as we found out, while it could be possible that adjuvants such as CFA or poly-IC might have a greater beneficial role at inducing tolerance in Th1-mediated immune diseases. This could be an area for further investigation, especially since the beneficial effect of adjuvants for tolerance induction may not be restricted solely to anti-CD4.

Prior studies have shown a clear role of Foxp3$^+$ Treg cells in transplantation tolerance induced with anti-CD4 [39, 340, 356]. Unexpectedly, we found that tolerance induction to soluble proteins was independent of Foxp3. Systemic delivery of a protein in alum appears to induce a state of immune tolerance dependent on IL-10 and not on Foxp3$^+$ Tregs. These findings question the reason for the absolute requirement of Foxp3$^+$ Treg cells in transplantation tolerance [356], as the same clone of non-depleting anti-CD4 MAb leads to Foxp3-independent immune tolerance in hemophilia, and possibly in allergy. These results could explain the apparently contradictory observations from transplantation studies using anti-CD4 for tolerance induction: while some studies where a skin graft alone was used in the tolerance-inducing protocol appeared to be IL-10-independent and Treg dependent [158, 340]; other studies where donor specific transfusion was administered in advance of skin transplantation appeared to rely on both Treg cells and IL-10 [351, 353]. Our data show that a protocol for tolerance induction that relies on IL-10 is sufficient to induce a state of tolerance robust enough to allow acceptance of a skin graft expressing the very same protein the subject was tolerized to.

One could speculate that the cytokine/ inflammatory environment in which tolerance is induced with an agent like anti-CD4 can influence the type of regulatory mechanisms (namely the nature of induced regulatory cells) that are generated. This issue, however, remains to be clarified. In any case the anatomic distribution of the same antigen – provided as part of a skin transplant or delivered systemically – can induce different mechanisms that have in common the maintenance of long term dominant tolerance.

It has been shown that Tregs can produce IL-10 in vivo [401, 402]. Indeed both $Foxp3^+$ or $Foxp3^-$ thymic precursors can give rise to IL-10 producing cells [403]. Only $Foxp3^-$ thymic precursors can give rise to all types of subsets ($Foxp3^+IL\text{-}10^+$, $Foxp3^+IL\text{-}10^-$, $Foxp3^-IL\text{-}10^+$) through a mechanism that is TGF-β dependent and IL-10 independent [403]. $Foxp3^+IL\text{-}10^-$ Treg cells tend to accumulate in the secondary lymphoid organs, lung and liver, while $Foxp3^+IL\text{-}10^+$ and $Foxp3^-IL\text{-}10^+$, are mostly found in Peyer's patches, small and large intestine [402, 403]. Nevertheless in pathological situations such as asthma $Foxp3^-IL\text{-}10^+$ CD4 T cells with a regulatory function can be found in the lung [73]. The best described regulatory T cell type associated with a $Foxp3^-IL\text{-}10^+$ phenotype are the T regulatory type 1 cells (Tr1). These cells are develop from $CD4^+CD25^-$ T cells under the influence of tolerogenic dendritic cells [404, 405] and exert their regulatory function by the extensive release of IL-10 [406].

Our observations that IL-10 deficient mice fail to maintain long term tolerance to systemically-administered proteins and that we cannot find evidence for an increase in the frequency of $Foxp3^+$ Tregs, suggest it is possible to induce tolerance in the absence of Foxp3 when the antigen is administered together with alum and anti-CD4. Indeed we confirmed this

hypothesis using Foxp3-deficient mice. Importantly, $CD4^+$ T cells exposed to CD4-blockade in presence of the antigen become endowed with regulatory properties (in spite of lacking Foxp3 expression). These Foxp3- regulatory cells are able to mediate dominant tolerance. We could not conclusively show that these Foxp3- regulatory cells are Tr1 cells, although this possibility appears as a likely hypothesis.

We confirmed that indeed tolerance induction to skin grafts, with anti-CD4 MAb treatment is dependent on $Foxp3^+$ Treg cells. In the same fashion, several animal models of autoimmunity can be successfully treated with anti-CD4, by a mechanism that appears to be Foxp3-dependent [357]. On the other hand, we confirmed in transgenic models that tolerization with delivery of proteins in alum together with anti-CD4 had no input of putative $Foxp3^+$ Tregs. In fact we were able to establish that there is a combination of a recessive mechanism where specific effector cells are eliminated (but only when antigen, alum and anti-CD4 are present) diminishing drastically the effector $CD4^+$ population (also known as AICD), while apparently a dominant mechanism outputs a T cell population that effectively suppresses the proliferation of naïve, antigen specific, T cells. Recessive mechanisms like AICD have been known to keep the balance between effector and regulatory mechanisms in immune tolerance [407]. Treatment with anti-CD3 MAbs, which have been used to prevent graft rejection [408, 409] and in clinical trials for the prevention of autoimmune diabetes [410], was shown to transiently deplete $CD4^+Foxp3^-$ effector T cells [275, 411] while $CD4^+Foxp3^+$ Treg cells show more resistance to this depletion [411] leading to an increase in frequency of regulatory cells. Also, in a GVHD model blockade of CD40-CD154 and CD80/CD86-CD28 interactions was shown to lead to peripheral elimination of host-reactive T cells [312, 412, 413]. In autoimmune encephalomyelitis

(EAE) anti-CD4 was also described to induce specific T cell depletion [384]. The deletion mechanism however remains unknown. Typically T cell death is induced by cell surface death receptors like Fas, or mitochondrial molecules such as Bim, Bad and Bax [414-417]. FcR-non-binding anti-CD3 MAb was reported to induce effector T cell death in a Fas, Bcl-2 and caspase-3 independent fashion [411]. We have also investigated the mechanism behind the specific deletion induced by anti-CD4 MAb through the evaluation of its impact in tolerance generation in Fas-deficient or Lck-driven Bcl-xl transgenic mice. We saw no attenuating impact in the tolerance induction in these mice models using anti-CD4 MAb (data not shown). However these results still need further confirmation and are very preliminary to draw any strong conclusion. Lastly we also analyzed the possible role for caspase-3, which is downstream of both intrinsic and extrinsic apoptosis pathway [417]. We found it was possible to induce tolerance to OVA-alum in a caspase-3 deficient mouse model. As before these results are too preliminary and remain to be further investigated. It seems there is enough evidence that the use of tolerogenic MAbs involves a mechanism of specific deletion of effector T cells, where, in some cases, there is a demonstrated association with a shift in the frequency of regulatory cells [409, 411]. It will be important to clarify the mechanism behind this T cell deletion that seems to be independent of the most obvious candidates (Fas, Bcl family and caspase-3). Activation-induced cell death in the periphery is associated with high intensity of TCR signaling [418], lack of cytokines such as IL-2 [419] or IL-12 [420] or impaired signaling at the immune synapse [421]. In vitro studies show that anti-CD4 induces a suboptimal activation [56] that renders $CD4^+$ T cells more susceptible to the effects of TGF-β in inducing $Foxp3^+$ Tregs [45, 56]. On the other hand $CD4^+$ T cells are described to need full interaction with MHC

class II molecules for the survival of resting CD4+ T cells in peripheral lymphoid organs [422] and that lack of CD4 expression on T cells increases their susceptibility to apoptosis leading to diminished survival [423]. In light of these facts it could be argued that CD4 blockade might be favoring the deletion of effector $CD4^+$ T cells, with cells in different stages of differentiation displaying distinct susceptibilities to apoptosis. It will be important to clarify what are the characteristics of T cell subsets that render them more susceptible or resistant to apoptosis, namely whether anti-CD4 indiscriminately deletes T cells or if (like anti-CD3) shows a particular preference in depleting recently activated T cells and spares $Helios^+$ Tregs [411].

In recent decades there was a remarkable increase in the understanding of the mechanisms that underlie immune system function. This has become an opportunity for the development of rational therapies for immune mediated diseases. The use of biological drugs, namely monoclonal antibodies, that can have a direct targeting of key molecules involved in the differentiation and behavior of immune cells has become common treatments in many diseases [328], The full potential of MAbs in inducing immune tolerance and their effect on primed cells remains largely an unaddressed medical need.

The work of this thesis focuses on the induction of immune tolerance created by the use of non-depleting anti-CD4 MAb. It also presents the need of adequate adjuvants as potential tolerance facilitators. The findings in this work also propose the notion that the inflammatory environment can indeed lead to the induction of different regulatory mechanisms.

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Annexes

List of Publications during the thesis

1. **Agua-Doce** A*, Caridade M*, Oliveira VG, BergmanL, Curotto de Lafaille M, Lafaille JJ, Demengeot J, Graca L. Induction of Foxp3-indepedent dominant transplantation tolerance *In preparation* * both authors contributed equally for this manuscript
2. **Agua-Doce A***, Oliveira VG*, Maria Curotto de Lafaille, Juan J. Lafaille, and Luis Graca. Adjuvant facilitates anti-CD4 mediated immune tolerance to recombinant factor VIII in hemophilia through a Foxp3-independent mechanism that relies on IL-10. *Accepted in Blood* * both authors contributed equally for this manuscript
3. Monteiro M, Almeida CF, **Agua-Doce A**, Graca L. Induced IL-17-Producing Invariant NKT Cells Require Activation in Presence of TGF-β and IL-1β. J Immunol. 2013 Jan 15;190(2):805-11
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Adjuvant facilitates tolerance induction to factor VIII in hemophilic mice through a Foxp3-independent mechanism that relies on IL-10

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Adjuvant facilitates tolerance induction to factor VIII in hemophilic mice through a Foxp3-independent mechanism that relies on IL-10

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Key Points

- An adjuvant (alum), known to boost immune responses, can be used to facilitate a tolerogenic protocol.
- Non-depleting anti-CD4 can lead to Foxp3+ Treg-independent tolerance that relies on IL-10.

Abstract

Current treatment for hemophilia consists in the administration of recombinant clotting factors, such as factor VIII (FVIII). However, patients with severe hemophilia can mount immune responses targeting therapeutically administered FVIII through inhibitory immunoglobulins that limit treatment efficacy. Induction of immune tolerance to FVIII in hemophilia has been extensively studied but remains an unmet need. We found that non-depleting anti-CD4 monoclonal antibodies (MAb) are effective in inducing long-term tolerance to FVIII in different strains of hemophilic mice. Tolerance induction was facilitated when anti-CD4 MAb were administered together with FVIII adsorbed in an adjuvant (alum). The observed state of tolerance was antigen specific, with mice remaining immune competent to respond to different antigens. Importantly, we found that following immunization with FVIII, the primed cells remained susceptible to tolerance induction. Studies with Foxp3- and IL-10-deficient mice demonstrated that the underlying tolerance mechanism is Foxp3-independent but requires IL-10. Our data show that an adjuvant when administered together with a tolerizing agent, such as non-depleting anti-CD4, can facilitate induction of long-term tolerance to recombinant proteins, possibly not only in hemophilia but also in other diseases that are treated with potentially immunogenic therapeutics.

Introduction

For many patients with severe hemophilia, replacement therapy with recombinant clotting factors represents an immune challenge with non-self proteins thus resulting in an immune response. As a consequence, immunoglobulins targeting infused clotting factors can lead to their inhibition (those immunoglobulins are termed inhibitors)[1]. Several approaches for immune tolerance induction (ITI), leading to the control of the production of clotting factor inhibitors, are currently used in hemophilia patients[2]. However, the success of ITI is not absolute and currently, at a time when viral contaminants in therapeutic products are a problem of the past, inhibitors became a major complication for hemophilia treatment. This has led to an increasing interest in developing new protocols for the induction of tolerance to therapeutically administered clotting factors[3]. Recent clinical trials have explored the putative higher tolerogenicity of factor VIII (FVIII) coupled with von Willebrand Factor (vWF), or B cell depletion with an anti-CD20 monoclonal antibody (MAb) rituximab[4,5]. The outcome of those trials has shown, at the most, a modest improvement in ITI efficacy. Consequently, those ITI approaches were not generally adopted and the development of better strategies for ITI remains an unmet medical need[6].

In recent years several strategies have been developed to promote immune tolerance towards alloantigens. Several studies have demonstrated that non-depleting monoclonal antibodies (MAbs) targeting T cell surface molecules are effective in achieving immune tolerance in transplantation and autoimmunity[7,8]. There is, however, a contrast between the efficacy in achieving long-term tolerance with MAbs such as CD4 or CD40L administered at the time of transplantation in pre-clinical studies[9-11], with a transient effect observed when the same reagents are used to induce tolerance to human recombinant factor VIII (hFVIII) or factor IX (hFIX) in mice[12,13]. Remarkably, when the recombinant clotting factors are expressed in tissues, for instance following viral transduction, tolerance induction appears to be easier to achieve[14].

These observations prompted us to investigate whether the difference between both outcomes is a consequence of differences in the way the antigen is presented to T cells for tolerance induction. It is plausible that efficient imposition of tolerance *in vivo*, would require an impact on a large proportion of the antigen-specific T cells, or stronger co-stimulation, something that can only be achieved in the presence of inflammation (namely in transplantation or viral transduction) or when an adjuvant is used.

We found that the most clinically relevant adjuvant – aluminium hydroxide (alum) – facilitates the induction of tolerance to hFVIII in mice. Surprisingly, the mechanism underlying tolerance

induction appears to be different from antibody-induced transplantation tolerance: while non-depleting anti-CD4 induces Foxp3^{+} regulatory T cells that are indispensable for transplantation tolerance, we were able to induce tolerance to foreign proteins in Foxp3-deficient mice. On the contrary, tolerance induction is critically dependent of IL-10, as it cannot be established in IL-10$^{-/-}$ mice.

Adjuvants can therefore have two opposing roles: they can contribute to boost immune responses and to overcome natural tolerance, but they can be instrumental in facilitating therapeutically induced tolerance when in presence of appropriate tolerance-inducing reagents.

Materials and methods

Animals. BALB/c, C57Bl/6, B6.IL-10$^{-/-}$, B6.Rag2$^{-/-}$, and DO11.10.Rag2$^{-/-}$, mice were maintained under specific pathogen-free (SPF) conditions. Hemophilia-A mice (HemA) with a targeted disruption of exon 16 in the FVIII gene[15], backcrossed into C57Bl/6 and BALB/c background, were provided by Professor David Lillicrap (Kingston, Canada). T/B monoclonal mice (T-Bmc, 17/9 DO11.10.RAG1$^{-/-}$) bear monoclonal T and B lymphocytes specific for ovalbumin (OVA) 323–339 and HA of influenza virus, respectively[16]. Procedures were approved by Direção Geral de Veterinária (Lisbon).

Animals were exposed to 1U hFVIII (Kogenate, Bayer, Germany), or to 20 µg OVA (Sigma, St Louis, USA) sometimes adsorbed in 2.0 mg endotoxin-free aluminum hydroxide (Alu-gel-S, Serva, Heidelberg, Germany).

Monoclonal antibodies. Non-depleting anti-CD4 (YTS177.9)[17], the isotype control (YKIX302), anti-CD25 (PC61), anti-IL-10R (1B1.1), and anti-TGFβ (1D11) were produced in house.

Quantification of immunoglobulins and cytokines. hFVIII-specific, OVA-specific, and HA-specific IgG1 were measured by ELISA using IgG1-HRP (SouthernBiotech, Birmingham, USA) with anti-hFVIII IgG1 standard (Abcam, Cambridge, UK), anti-OVA IgG1 standard (Serotec, Oxford, UK) and anti-HA IgG1 standard (Sigma). The ELISA assay for IL-10 measurement was performed using a kit (Peprotech, London, UK).

Flow cytometry. Cells were stained with CD25-PE-Cy7 (PC61.5), Foxp3-APC (FJK-16s), CD3-PE-Cy7 (145-2C11), CD4-PerCp (RM4-5), and DO11.10 TCR-specific KJ1-26-PE. Six color analyses were performed using a LSR-Fortessa (BD Bioscience).

Bethesda assay. Inhibitory FVIII Abs were measured using the Bethesda assay and reported as Bethesda units/mL as previously described[18]. Briefly, mouse plasma was serially diluted (Technochrom® FVIII:C, Technoclone, Austria) such that the residual FVIII:C for each sample was between 25% and 75%, mixed 1:1 with pooled normal human plasma (Factor VIII Inhibitor reagent Kit), and incubated for 2 hours at 37°C. The remaining FVIII activity was quantified using a chromogenic assay (Technochrom). To reduce error in the assay and inconsistency between samples, the reported inhibitor titer in BU/mL was calculated from the dilution of plasma that yielded a residual FVIII:C of approximately 50%. By definition 1 BU/mL is defined as the dilution of plasma containing FVIII inhibitory activity that results in 50% inhibition of FVIII activity (FVIII:C).

Statistical analysis. Statistical significance was determined using the two-tailed non-parametric Mann-Whitney U test and values of $P<0.05$ were deemed significant.

Results

hFVIII alum increases the tolerogenicity of anti-CD4 MAb.

Alum has been used as an adjuvant in the formulation of human vaccines since 1926[19,20]. We treated BALB/c and C57Bl/6 mice with hFVIII or hFVIII-alum together with non-depleting anti-CD4 at the indicated time points (**Figure 1a**). Serum anti-hFVIII IgG1 was measured following additional administrations of hFVIII. Our results confirmed that anti-CD4 treatment only has a moderate impact in preventing the generation of inhibitor antibodies to hFVIII (**Figure 1b, c**). However, when hFVIII-alum was used at the time of anti-CD4 treatment the concentration of hFVIII-specific antibodies remained significantly reduced even following multiple administrations of hFVIII, up to 110 days following the initial treatment. Note that hFVIII-alum is, as anticipated, highly immunogenic leading to high levels of anti-hFVIII immunoglobulins when used in the absence of anti-CD4. We also quantified hFVIII-specific IgG2a and IgG2b that remained negligible in both strains (Supplemental Figure 1).

Next we investigated whether the route of hFVIII-alum administration could influence tolerance induction. C57Bl/6 mice were exposed to hFVIII-alum i.p. (as described above), intramuscular or subcutaneous. Subsequent administrations of hFVIII were performed i.v. (**Figure 1d**). We found that tolerance to hFVIII could be induced regardless of the route of administration.

The “depot effect” has been regarded as a possible mechanism by which alum exerts its adjuvanticity[21]. According to this effect, adsorption of hFVIII in Alum would confer a slow

release and consequently an increased half-life of hFVIII *in vivo* thus providing stable and long-term antigen presentation. This hypothesis is especially relevant as it has been described that sustained release of peptides, by an implanted osmotic pump, can lead to immune tolerance[22]. We addressed this issue by repeatedly injecting hFVIII every other day for two weeks in presence of anti-CD4 MAb. We found that animals exposed to multiple administrations of hFVIII+anti-CD4 still produced higher titers of anti-hFVIII IgG1 than animals treated with hFVIII-alum+anti-CD4 (**Figure 1e**).

Although the anti-CD4 MAb has a non-depleting isotype, we confirmed it does not lead to direct T cell depletion *in vivo* by treating DO11.10.Rag2$^{-/-}$ mice with anti-CD4. This way we could circumvent the coating of the CD4 molecule, being able to accurately quantify CD4$^+$ T cells by staining their transgenic TCR (identified with KJ26.1 MAbs). We found that the frequency of CD4$^+$ splenocytes at day 7 following anti-CD4 treatment remained unchanged (**Figure 1f**).

Tolerance induction requires IL-10 and is independent of Foxp3$^+$ Treg cells.

We and others have shown that transplantation tolerance can be induced with non-depleting anti-CD4 MAb, in a process that is Foxp3$^+$ Treg cell-mediated and TGFβ-dependent[9,23-26]. In some conditions, IL-10 appears to play a role in transplantation tolerance[24,27]. In order to study the mechanism by which anti-CD4 MAb induces tolerance in hFVIII-alum treated animals, we used MAbs to block TGF-β or IL-10R at the time of treatment. In addition, another group of animals was treated with depleting anti-CD25 MAb prior to treatment. We found IL-10R-blockade during the initial two weeks had a dramatic effect in preventing tolerance induction (**Figure 2a**). However, only a modest increase of anti-hFVIII IgG1 was observed in mice subjected to depletion of CD25$^+$ T cells or to neutralization of TGF-β.

These data suggest Foxp3$^+$ Treg cells may only have a minor role in mediating tolerance in our model. In fact, no differences were found in percentages or absolute numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells in the spleen of animals treated with hFVIII-alum + anti-CD4 when compared to the positive group (hFVIII-alum + isotype) 90 days after the induction of tolerance (**Figure 2b and 2c**).

As experimental mice have a polyclonal T cell repertoire, putative changes in the frequency of antigen-specific Treg cells may be beyond the limit of detection. In addition, CD25-depletion with PC61 is known to spare a significant number of Foxp3$^+$ Treg cells[28]. We therefore used Foxp3-deficient mice to directly assess if tolerance to a foreign protein could be induced in the

absence of Foxp3^{+} Treg cells. Since Foxp3-deficiency leads to severe autoimmunity we had to perform this experiment in mice devoid of autorreactive T cells. We used T/B Foxp3^{sf} monoclonal mice (T-Bmc FoxP3^{sf}), characterized by monoclonal populations of T and B lymphocytes specific, respectively, for $OVA_{323–339}$ and HA[16]. As a consequence, we had to use a cross-linked protein of OVA-HA as a model antigen instead of hFVIII. Treatment of T-Bmc mice with OVA-HA-alum and anti-CD4 was equally effective in the presence or absence of functional Foxp3 (**Figure 2d**).

To further confirm the key role of IL-10 in inducing tolerance to hFVIII, we treated IL10$^{-/-}$ mice with hFVIII-alum + anti-CD4. We confirmed it was not possible to prevent generation of anti-hFVIII IgG1 in IL10$^{-/-}$ mice by treatment with hFVIII-alum + anti-CD4 (**Figure 2e**).

Taken together, our data show that induction of tolerance to hFVIII-alum in the presence of anti-CD4 MAb is IL-10-dependent.

Non-depleting anti-CD4 induces long-term prevention from inhibitor formation in hemophilia A mice.

To determine if treatment with anti-CD4 could prevent the formation of FVIII inhibitors in hemophilia, we used mice deficient in FVIII (hemophilia A mice, HemA). C57Bl/6 HemA mice treated with anti-CD4 exhibited low serum concentration of anti-hFVIII IgG1 for 90 days, even after multiple exposures to hFVIII (**Figure 3a-b**). In addition, we used the Bethesda Assay to measure the titer of FVIII inhibitors at day 90. We found that anti-CD4 treatment successfully prevented the formation of inhibitors in HemA mice (**Figure 3c**). Similar results were obtained with BALB/c HemA mice (**Figure 3d-e**). We also found that hFVIII-exposed HemA mice produced high titres of hFVIII-specific IgG2a and IgG2b, unless treated with anti-CD4 MAb (Supplemental Figure 1). Our findings on the different isotype preferences by the two strains of HemA mice are consistent with previous reports[29].

We confirmed tolerance induction could also be achieved in HemA mice when hFVIII-alum was administered s.c. and the anti-CD4 MAb i.v. (**Figure 3f**).

We also found that splenocytes collected from HemA mice treated with hFVIII-alum + anti-CD4 when stimulated *in vitro* in presence of anti-CD3 were more prone to produce IL-10 than splenocytes from naïve non-manipulated mice, or mice treated with an isotype control (**Figure 3g**).

Tolerant mice remain immune competent

Although the anti-CD4 Mab clone has a non-depleting isotype (i.e. it does not directly lead to T cell lysis), we confirmed that mice treated with anti-CD4 remained immune competent. We used C57Bl/6 mice treated with anti-CD4 as described in Figure 1, but using hFVIII-alum or OVA-alum as the "tolerizing antigen" administered at the time of Mab treatment. We waited five weeks to minimize the potential persistence of antigen or anti-CD4. At day 50 and 64, the mice were exposed to the same or to the other antigen (**Figure 4a**). We found that treatment with anti-CD4 Mab prevented subsequent immunization to the same antigen without suppressing immune responses to the other antigen (**Figure 4b and 4c**). In other words, mice "tolerized" with OVA-alum could respond to subsequent immunization with FVIII-alum, but did not respond to OVA-alum. Conversely, mice "tolerized" with FVIII-alum did not respond to a later challenge with FVIII-alum, but were fully competent to produce antibodies following immunization with OVA-alum.

These results show that treatment with anti-CD4 does not induce global immune suppression, but rather antigen-specific unresponsiveness.

Anti-CD4 Mab is effective in inducing tolerance following sensitization

Unlike autoimmunity or allergy, in hemophilia there is an opportunity to induce tolerance in advance of immune sensitization, as the first exposure to the antigens is controlled by the clinician. However, since many hemophilia patients do not develop inhibitors, ITI strategies are often required when the patient is already sensitized. Previous studies suggested that CD4-blockade may be effective in sensitized hosts[30]. But hFVIII-specific IgG produced at the time of sensitization, given their long half-life, may obscure the eventual effectiveness of tolerance induction at a later time.

To overcome this limitation, and since it is not feasible, in mice, to remove pre-existing FVIII-specific IgG by plasmapheresis, we developed an experimental setting to address the role of CD4-blockade in pre-sensitized cells in the absence of preformed FVIII-specific IgG (**Figure 5a**).

In brief, C57Bl/6 mice were immunized with hFVIII-alum at day 1 and 14, and sacrificed at day 30. This immunization protocol leads to high concentration of hFVIII-specific IgG1 (198.0 ± 14.7 µg/ml). We collected and pooled T and B cells from spleen, pooled lymph nodes, and bone marrow. The lymphocytes were then adoptively transferred i.v. into congenic $Rag2^{-/-}$ or C57Bl/6 recipients. The recipient mice were then treated with hFVIII-alum + anti-CD4 Mab, and exposed to additional challenges with hFVIII (**Figure 5a**).

Using Rag2$^{-/-}$ recipients we confirmed that the adoptively transferred primed T and B cells were sufficient to produce anti-hFVIII IgG1 (**Figure 5b**). However, treatment with anti-CD4 was effective in preventing the production of hFVIII-specific IgG1 by those primed cells (**Figure 5b**).

The adoptive cell transfer into C57Bl/6 mice showed that the adoptively transferred cells were indeed primed. Our data show that recipients of primed cells displayed a rapid and high magnitude secondary type immune response following exposure to hFVIII, when compared with control mice that did not receive primed cells, and that displayed a primary response (**Figure 5c**). We also observed that when the primed cells were transferred into C57Bl/6 mice, treatment with anti-CD4 Mab prevented production of anti-hFVIII IgG1 (**Figure 5c**).

We then investigated whether hFVIII-alum + anti-CD4 could protect mice sensitized to hFVIII from further production of FVIII-specific antibodies. We found that animals exposed to hFVIII when subsequently treated with hFVIII-alum (i.p. or s.c.) + anti-CD4 were protected from an increase in serum levels of FVIII-specific IgG1, in spite of repeated administrations of hFVIII (**Figure 5d**). The effect was not due to non-specific effects of anti-CD4, as mice treated with anti-CD4 in the absence of hFVIII responded to subsequent exposure to hFVIII with production of hFVIII-specific IgG1.

These results demonstrate that anti-CD4 Mab treatment is effective in controlling the response to hFVIII in pre-sensitized animals.

Discussion

Our results show that an adjuvant – alum – can enhance the ability of non-depleting anti-CD4 MAb to achieve tolerance to hFVIII. These results can appear counterintuitive as usually adjuvants, such as alum, are used to boost immunity namely leading to production of protective antibodies. It is likely that when tolerance is based on CD4-blockade it will be necessary that the majority of antigen-specific T cells will be exposed to the antigen during the time when the CD4-blocking MAb is present, and with optimal co-stimulation. In this regard, our results on the antigen-specificity of tolerance (Figure 4) also confirm that tolerance is imposed on the lymphocytes that are engaged in antigen recognition at the time of CD4-blockade: our data show that exposure to hFVIII-alum + anti-CD4 can prevent subsequent immune responses targeting hFVIII, while allowing successful immunization to an unrelated antigen (OVA). Similar results were obtained in the converse experiment: when tolerance is induced with OVA-alum + anti-CD4, the animals can produce antibodies targeting hFVIII, following exposure to the clotting factor. We can conclude that the initial exposure to hFVIII-

alum is therefore critical to control the FVIII-specific T cell clones, but does not affect T cell clones with other specificities.

Given previous reports showing a clear role of Foxp3$^+$ Treg cells in transplantation tolerance induced with anti-CD4[9,26,31], it was surprising to find that tolerance induction to proteins in alum could be achieved in the absence of Foxp3 (Figure 2). We have confirmed that indeed tolerance induction to skin grafts, induced following anti-CD4 MAb treatment, is dependent of Foxp3$^+$ Treg cells (data not shown). In the same fashion, several animal models of autoimmunity can be successfully treated with anti-CD4, by a mechanism that appears to be Foxp3-dependent[32]. Inhibition of mTOR has been shown to facilitate the induction of Foxp3$^+$ Treg cells, and a strategy to improve the efficacy of induction of transplantation tolerance[33]. It was also reported that treatment with rapamycin (a mTOR inhibitor) can lead to tolerance to FVIII[34].

Our data show that the systemic distribution a protein in alum appears to take advantage of an IL-10-dependent and Treg-independent mechanism. This observation may explain the apparently contradictory results from transplantation studies using anti-CD4 for tolerance induction: while studies where a skin graft alone is used tolerance appears to be IL-10-independent and Treg dependent[9,35]; but studies where donor specific transfusion and a skin graft are used appear to rely on both Treg cells and IL-10[24,27], suggesting that systemic distribution of the antigen may recruit a IL-10-mediated tolerogenic mechanism. It remains to be established the reason for the absolute requirement of Foxp3$^+$ Treg cells in transplantation tolerance[31], while the same clone of non-depleting anti-CD4 MAb leads to Foxp3-independent immune tolerance in hemophilia.

By neutralizing IL-10R *in vivo* during the first two weeks of anti-CD4 treatment we could infer the participation of IL-10 in the mechanism leading to tolerance, but we could also establish the importance of that initial period for tolerance induction (Figure 2). This observation is consistent with the need to impose tolerance in antigen-specific cells during a window of time, after which the immune system regains the ability to respond to foreign antigens, as discussed above. In addition, by showing that repeated administrations of hFVIII (without alum) during those initial two weeks did not lead to long-term tolerance, as we observed when hFVIII-alum was used (Figure 1C), allowed us to exclude that tolerance facilitation by alum could be simply due to the "depot effect" leading to greater half-life of the antigen.

A usual concern in transplantation tolerance experiments is the influence of the genetic background in the results[36]. Some strains, namely C3H and CBA/Ca are known to be more

permissive to the induction of transplantation tolerance than other strains like C57Bl/6 and BALB/c. In order to evaluate whether tolerance to hFVIII was strain-specific, we confirmed that long-term tolerance to hFVIII could be achieved in C57Bl/6 and BALB/c mice, and in mice from those two strains deficient in FVIII (HemA mice). When we used hemophilic mice we could directly document the physiological impact of FVIII inhibitors, not only by their quantification using the Bethesda assay but also by our subjective assessment of bleeding from those mice. Treatment with hFVIII-alum + anti-CD4 led to long-term protection from the generation of inhibitors in the two strains of hemophilic mice.

Several other MAbs targeting T cell co-receptors (namely anti-CD3) and co-stimulatory molecules have been shown effective in inducing immune tolerance in transplantation, autoimmunity, and allergic diseases[8,37]. Recently it was reported that antibodies targeting CD3 can induce long-term tolerance to FVIII in hemophilic mice[38]. In addition, MAb targeting ICOS are effective in inducing long term tolerance to hFVIII in mice, following gene therapy[14]. Targeting B cells with an anti-CD20 IgG1 is another approach that could lead to tolerance to FVIII[39]. Concerning non-depleting anti-CD4 there is, however, one special case of successful induction of long-term tolerance to foreign proteins in the absence of adjuvant – it is when the foreign protein is an immunoglobulin. In fact in some of the earliest demonstrations that non-depleting anti-CD4 can induce tolerance to foreign antigens, immunoglobulins (Ig) from different animal species were used as the tolerizing antigen[40,41]. It is likely that the special tolerogenicity of foreign Ig compared with FVIII represents the fact that antigen presenting cells can capture foreign Ig with specific Fc receptors for efficient presentation.

Given the promising pre-clinical results, anti-CD4 MAb was among the very first MAb to enter clinical trials[42,43]. The outcome of those early clinical trials was disappointing, which led to the search for alternative tolerance-inducing strategies. In retrospect, however, it is possible that the low efficacy was due to immaturity of the field. In fact, clinical trials in patients with rheumatoid arthritis and multiple sclerosis were not performed with humanized MAb, and the low efficacy correlated well with the generation of Ig targeting the therapeutic MAb[44]. In addition, a well known clinical trial in asthma a different anti-CD4 MAb was used (keliximab) that caused significant T cell depletion[45]. More recently, pre-clinical studies with a humanized non-depleting anti-CD4 have shown it could induce tolerance in non-human primates to foreign Ig[46]. It is possible that the use of alum as an adjuvant, as we now describe, will allow tolerance induction to hFVIII but also to other therapeutic recombinant proteins, namely in enzyme replacement therapy. A potential limitation for the clinical application of this approach

is the perceived risk of inducing further production of FVIII inhibitors with the use of an adjuvant (alum).

It is well established that the primed immune system is more resistant to tolerance inducing strategies. Nevertheless, most clinical situations would require tolerance induction in an immunized individual. In hemophilia, FVIII inhibitors are only produced in a proportion of patients. Therefore, tolerance induction should only be initiated in the patients that would require this intervention, and those patients can only be identified after the onset of inhibitors. However, the study of tolerance inducing strategies in mice previously immunized to hFVIII has been difficult due to the long half-life of hFVIII-specific IgG – the experimental readout. As a consequence, most studies in mice are restricted to the ability to induce tolerance at the time of the initial exposure to hFVIII – an event that is controlled by the clinician. We developed an experimental system to study the effect of hFVIII-alum + anti-CD4 on the primed immune system (Figure 5). Instead of removing pre-existent hFVIII-specific IgG by plasmapheresis – something that we could not do in mice – we transferred primed immune cells to a new host. This way it became possible to confirm that our protocol could prevent *de novo* production of hFVIII-specific antibodies by the primed cells. Such adoptive cell transfer system is unlikely to reproduce in its entirety the characteristics of a primed immune system. As a consequence, we confirmed that mice exposed to hFVIII (bearing hFVIII-specific IgG1) when treated with hFVIII-alum + anti-CD4 were protected from further production of of hFVIII-specific antibodies following subsequent hFVIII infusions. These findings are consistent with our previous report that anti-CD4 is able to prevent allergic airways disease in mice pre-sensitized to an allergen (house dust mite)[30]. Furthermore, a report has shown that MAbs targeting ICOS and CD154 can prevent memory plasma cells to respond to FVIII[47], and treatment of FVIII-primed mice with B cells expressing the immunodominant FVIII C2 and A2 domains was shown to lead to humoral tolerance[48].

Taken together our results show that alum, in spite of its common use to boost immune responses, can be effective in enhancing the ability of non-depleting anti-CD4 MAb to induce long-term and antigen-specific tolerance to hFVIII in mice. The tolerogenic mechanism relies on IL-10 and is independent of Foxp3$^+$ Treg cells. We believe that the use of alum, or other adjuvants, may constitute a strategy that could be applied to different immunogenic therapeutic proteins and other tolerance-inducing MAbs. In this case, our findings may be of interest beyond the field of hemophilia, namely in regard to enzyme replacement therapy.

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Authorship

V.G.O. and A.A-D designed and performed research, analyzed data, and wrote the paper; M.C.L. and J.J.L. contributed vital new reagents and analyzed data; L.G. designed research, analyzed data, and wrote the paper.

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Figure Legends

Figure 1. *hFVIII-alum increases the tolerogenicity of anti-CD4 Mab.* (A) C57Bl/6 and Balb/c mice were treated with 1 U hFVIII or HFVIII-alum and 2 x 1 mg non-depleting anti-CD4 (or isotype control) on the indicated days, followed by subsequent administrations of 1 U hFVIII as represented. The presence of serum hFVIII-specific Ig was investigated at days 30, 60, and 90 or 110 following last administration of anti-CD4. (B) Serum concentration of hFVIII-specific IgG1 in C57Bl/6 mice (n=6; **: $p<0.01$), and (C) in Balb/c mice (n=6; *: $p<0.05$; **: $p<0.01$). (D) hFVIII-alum and anti-CD4 were administered through different routes (as indicated in the figure label) according to the schedule represented in (A). One group of mice was treated with an isotype control. All mice were then exposed to hFVIII i.v. and serum anti-hFVIII IgG1 was quantified. Animals that received hFVIII-alum together with anti-CD4 remained tolerant regardless of the administration route (n=6; $p<0.001$). (E) Persistent serum levels of hFVIII did not prevent the generation of hFVIII-specific IgG1 in presence of anti-CD4. C57Bl/6 mice were treated with anti-CD4 MAbs in the days indicated above. Some mice were treated with 1 U hFVIII every other day for two weeks. We found that animals exposed to multiple administrations of hFVIII still produced higher titers of anti-hFVIII IgG1 than animals treated with hFVIII-alum (n=6; $p<0.001$). (F) Administration of the non-depleting anti-CD4 MAb (YTS177.9) does not lead to CD4 T cell depletion. DO11.10.Rag$^{-/-}$ mice were treated with 2 x 1 mg YTS177.9 and splenocytes were examined by flow cytometry for evidence of T cell depletion at day 7 (n=5; *p*, non-significant).

Figure 2. *Tolerance induction requires IL-10 and is independent of Foxp3^{+} Treg cells.* (A) Mice were treated with hFVIII-alum and anti-CD4 as described in Figure 1A. Different groups were also treated with the indicated MAbs. All mice were exposed to 1 U hFVIII on days 30 and 60. Serum concentrations of hFVIII-specific IgG1 were determined on days 30, 60, and 90 (n=5; **: $p<0.01$; ***: $p<0.001$). (B) Representative dot-plots and (C) quantification of splenic CD4^{+}CD25^{+}Foxp3^{+} T cells from animals treated with anti-CD4 MAb or an isotype control, at day 90 (n=5; *p*, non-significant). (D) T/B monoclonal mice (T-Bmc) T-Bmc Foxp3-deficient mice (T-Bmc FoxP3sf), were immunized on days 1 and 14 with OVA-HA-alum in the presence of non-depleting anti-CD4 or an isotype control. Serum HA-specific IgG1 was determined on day 24 (n=5; $p<0.001$). (E) IL-10$^{-/-}$ and IL-10$^{+/+}$ mice were treated with hFVIII-alum and anti-CD4 MAbs as described above. The serum concentration of hFVIII-specific IgG1 was quantified on day 90 (n=6; $p<0.001$). (A-E) Data are representative of two independent experiments.

Figure 3. *Non-depleting anti-CD4 induces long-term protection from inhibitor formation in hemophilia-A mice.* (A) HemA mice were treated with 1 U hFVIII or HFVIII-alum and 2 x 1 mg non-depleting anti-CD4 (or isotype control) on the indicated days, followed by subsequent administrations of 1 U hFVIII as represented. The presence of serum hFVIII-specific Ig was investigated at days 30, 60, and 90 following last administration of anti-CD4. (B) Quantification of serum hFVIII-specific IgG1 from C57Bl/6 Hem-A mice at day 30 and at day 90, following multiple exposures to hFVIII (n=8; $p<0.001$). (C) Quantification of FVIII inhibitors from the serum of C57Bl/6 mice at day 90 (n=8; $p<0.01$). (D) Serum hFVIII-specific IgG1 from BALB/c Hem-A mice (n=4; $p<0.01$). (E) Quantification of hFVIII inhibitors from BALB/c Hem-A mice (n=4; $p<0.05$). (F) C57Bl/6 HemA mice were treated on days 1 and 14 with hFVIII-alum (i.p. or s.c.) and anti-CD4 (i.p. or i.v.). A control group remained untreated. Subsequent challenges were done by i.v. administration of hFVIII, in a total of 4 injections, given twice weekly starting on day 21. Quantification of the anti-hFVIII IgG1 levels and Bethesda Units, measured one week after the final exposure to hFVIII (n=6; $p<0.05$). (G) Quantification of IL-10 concentration in supernatants of splenocytes from C57Bl/6 Hem-A mice, collected at day 7 following initial treatment with HFVIII-alum + anti-CD4. The splenocytes were stimulated with anti-CD3 for three days (n=3; $p<0.05$). Data are representative of two independent experiments.

Figure 4. *Non-depleting anti-CD4-treated mice remain immune competent.* (A) C57Bl/6 mice were treated as described previously with anti-CD4, but using FVIII-alum or OVA-alum as the "tolerizing antigen" administered at the time of Mab treatment. Five weeks following the last MAb infusion the mice were exposed to the same or to the other antigen. Control groups were not treated with anti-CD4. (B) Some mice were immunized with hFVIII-alum on days 50 and 64, and serum hFVIII-specific IgG1 was quantified at day 73. We found that unlike mice initially treated with OVA-alum + anti-CD4, mice treated with hFVIII-alum + anti-CD4 remained unresponsive to the subsequent immunization with hFVIII-alum (n=4; $p<0.05$). (C) Other mice were immunized at day 50 and 64 with OVA-alum, and serum OVA-specific IgG1 was quantified on day 73. Animals initially treated with hFVIII-alum + anti-CD4 remained competent to respond to immunization with OVA-alum, unlike mice initially treated with OVA-alum + anti-CD4 that remained tolerant to OVA (n=4; $p<0.05$). Data are representative of two independent experiments.

Figure 5. *Anti-CD4 Mab is effective in inducing tolerance following sensitization.* (A) C57Bl/6 mice were sensitized with hFVIII-alum at day 1 and 14, and sacrificed at day 30. Leukocytes from spleen, pooled lymph nodes, and bone marrow were collected and adoptively transferred

i.v. into congenic $Rag2^{-/-}$ or C57Bl/6 recipients. The recipient mice were then treated with hFVIII-alum + anti-CD4 Mab (or an isotype control) on the day of cell transfer and 14 days later (consistent with protocol represented in Figure 1A), and all groups of mice were subsequently challenged with 3x 1U hFVIII on days 10, 12, and 14 following the last administration of anti-CD4. (B) Quantification of serum hFVIII-specific IgG1 in $Rag2^{-/-}$ recipient mice. Animals that received primed T and B cells were competent to produce anti-hFVIII IgG1 (n=5; $p<0.001$), but antibody production was abrogated in mice treated with anti-CD4 (n=5; $p<0.001$; N.D., not detected). (C) Concentration of serum hFVIII-specific IgG1 in C57Bl/6 mice transferred with primed T and B cells. Animals adoptively transferred with primed cells produced higher levels of hFVIII-specific IgG1 than animals that did not received primed cells (n=5; $p<0.001$). Treatment with anti-CD4 prevented production of hFVIII-specific IgG1 by the primed lymphocytes (n=5; $p<0.001$; N.D., not detected). Data are representative of two independent experiments. (D) C57Bl/6 mice were sensitized with hFVIII on days 1, 5, 10, and 15, and treated with hFVIII-alum and anti-CD4 (i.p. or s.c.) on days 22 and 36. Control mice received anti-CD4 without hFVIII. One control group had not been previously exposed to hFVIII. All mice received hFVIII i.v. on days 54, 56, 64, 66, 74, and 76. Anti-hFVIII IgG1 was quantified on the indicated days. Mice treated with hFVIII-alum + anti-CD4 (regardless of the route of administration) were protected from production of anti-hFVIII IgG1 following subsequent exposure to hFVIII (n=6; $p<0.001$).

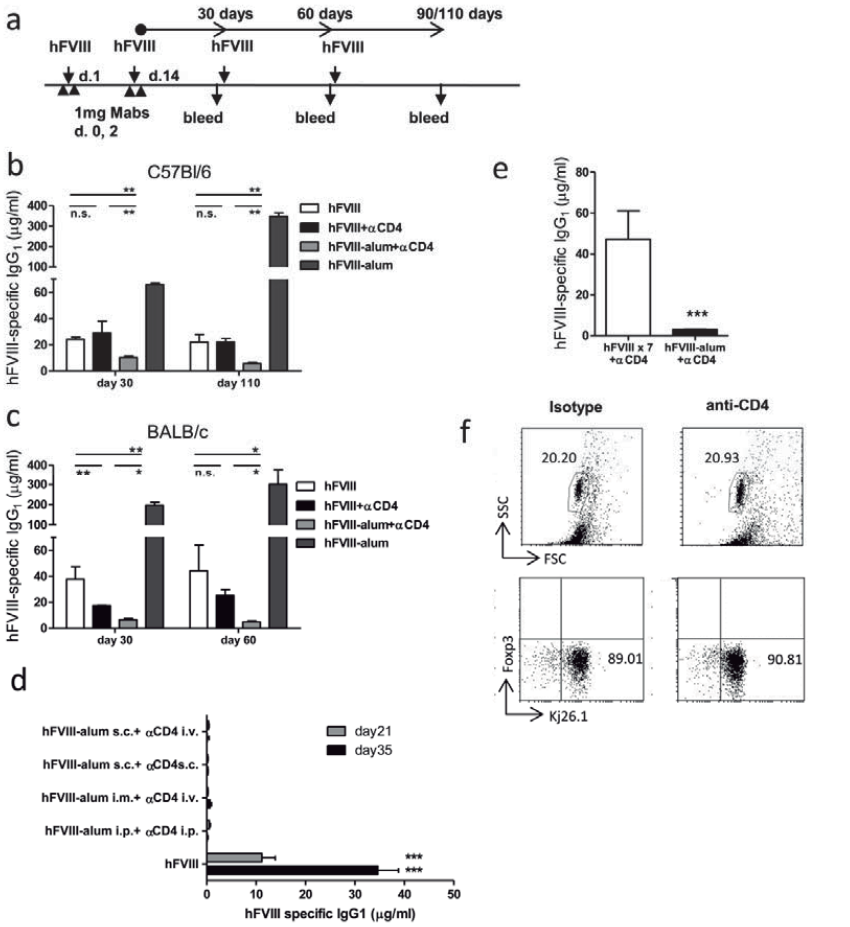
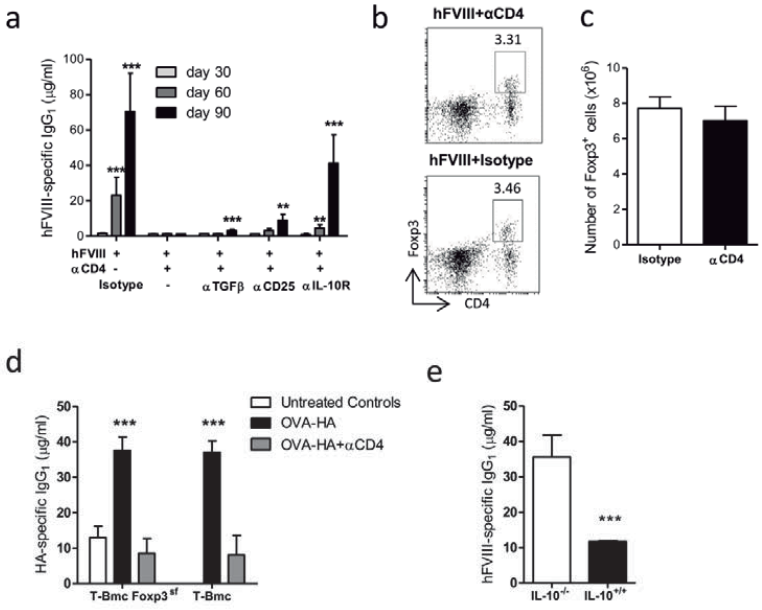


Figure 1



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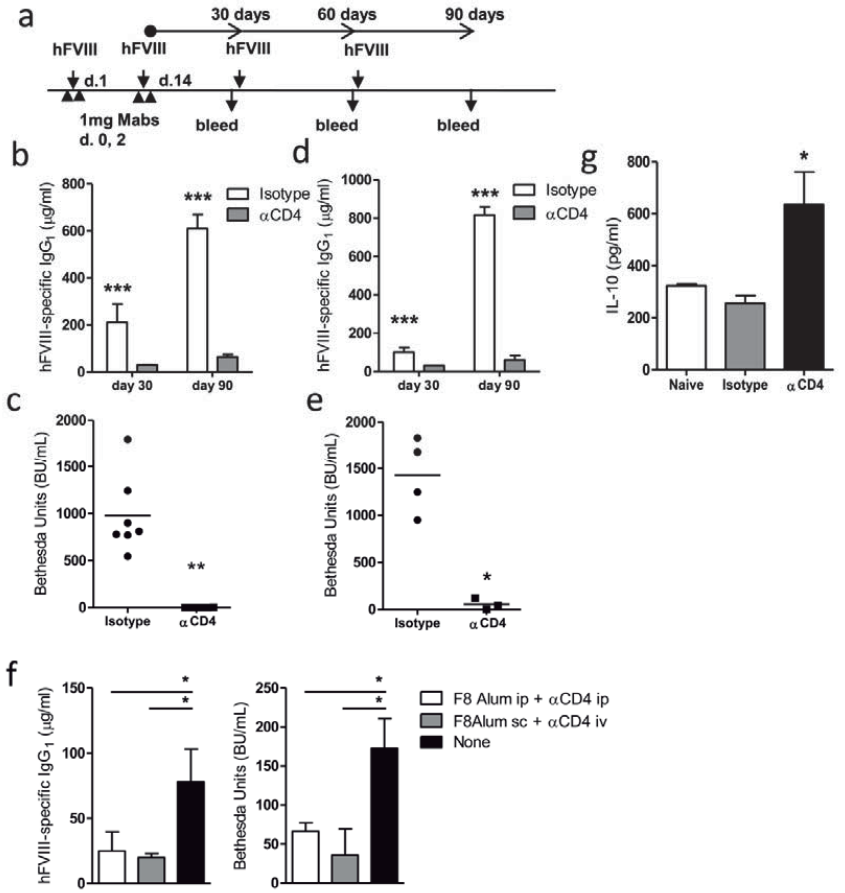

Figure 3

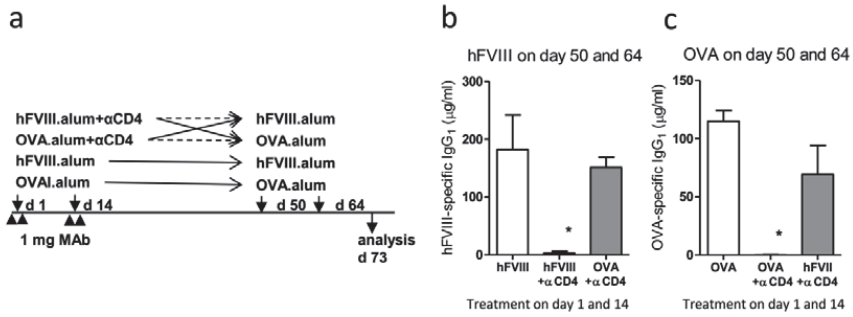

Figure 4

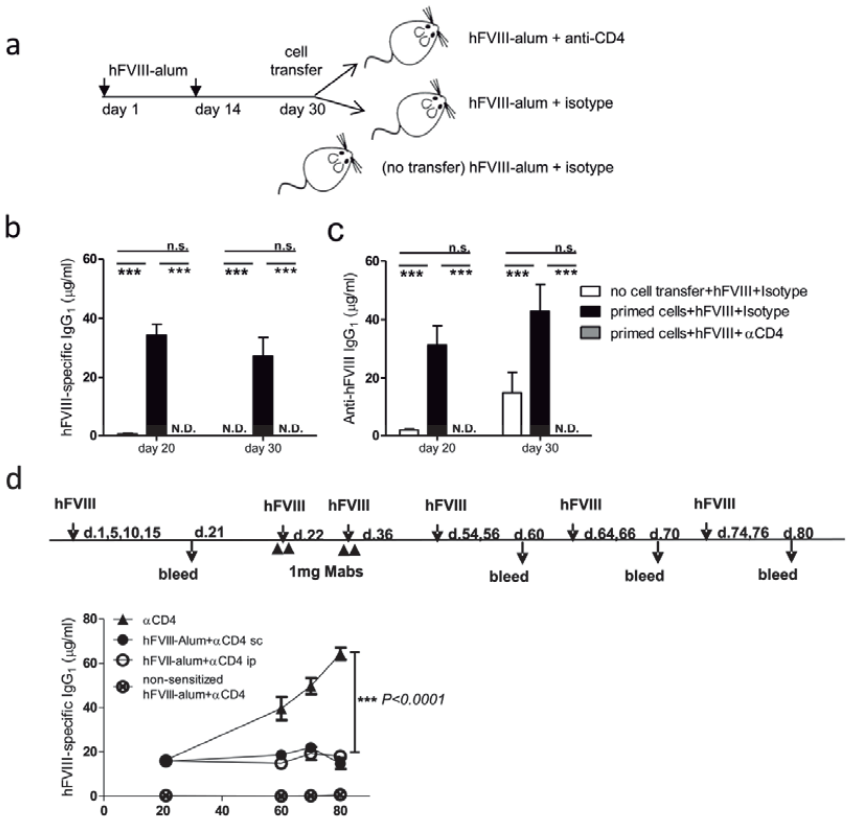

Figure 5

Induced IL-17–Producing Invariant NKT Cells Require Activation in Presence of TGF-β and IL-1β

Marta Monteiro, Catarina F. Almeida, Ana Agua-Doce and Luis Graca

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Induced IL-17–Producing Invariant NKT Cells Require Activation in Presence of TGF-β and IL-1β

Marta Monteiro,[1] Catarina F. Almeida,[1] Ana Agua-Doce, and Luis Graca

IL-17 production by innate-like lymphocytes, including γδ and invariant NKT (iNKT) cells, have been ascribed to specific lineages that are endowed with this functional specialization during thymic differentiation. IL-17–producing iNKT cells have been described as a $CD4^{-}NK1.1^{-}$ lineage in mice and $CD161^{+}$ in humans. We found that, in mice, noncommitted iNKT cells can be induced to produce IL-17 when activated in presence of TGF-β and IL-1β. This peripheral induction of IL-17 expression could be observed in any subset irrespectively of CD4 and NK1.1 expression, the process leading to loss of NK1.1 expression and partial CD4 downmodulation. Furthermore, induced IL-17–producing iNKT cells were sufficient to drive neutrophilic airways inflammation upon intratracheal adoptive cell transfer into congenic mice. Taken together, our data show that similarly to regulatory T cells, which have a natural and peripherally induced subset, IL-17 production by iNKT cells can also be imprinted in natural iNKT17 cells or peripherally induced. *The Journal of Immunology*, **2013, 190: 805–811.**

Invariant NKT (iNKT) cells are a subset of unconventional T lymphocytes that rearrange a limited repertoire of αβ TCR chains allowing the specific recognition of glycolipid Ags presented by CD1d molecules (1). These cells can be found in mice and humans, and display immune regulatory functions that may contribute to either protective or deleterious immune responses in several disease settings, including bacterial, viral, fungal, and protozoan infections, as well as in allergy and autoimmunity (2, 3).

In addition to the TCR, iNKT cells can express other receptors from the T cell lineage, like the CD4 coreceptor, as well as molecules typical of NK cells, such as NK1.1 (1). The differential expression of these particular molecules among iNKT lymphocytes has been widely used to discriminate distinct developmental stages in the thymus and mature iNKT cell subsets in the periphery (4–6). These peripheral iNKT cells exhibit an effector/memory phenotype even in the absence of prior stimulation. Its activated phenotype is acquired during thymic differentiation, leading to the postulation that iNKT cells could be selected and/or expanded by self-agonists in the thymus (7). Upon Ag encounter, iNKT cells rapidly secrete copious amounts of cytokines, influencing the recruitment and activation of several immune cells, including T and B lymphocytes, macrophages, DCs, NK cells, and neutrophils (8–10).

In the past few years, several groups have demonstrated that iNKT cells can also secrete IL-17 (4, 6, 11–14). This cytokine plays a key role in the recruitment, activation, and migration of neutrophils, and can act on a plethora of cell types to induce cytokine and chemokine secretion (15–19). Effector CD4 T cells that specialize in IL-17 secretion, termed Th17 cells, differentiate in the periphery after activation in presence of TGF-β, IL-6, and IL-1β produced mainly by innate cells, and rely on IL-23 and IL-21 for maturation and expansion after Th17 commitment (20–22). In contrast, IL-17–secreting iNKT cells are believed to belong to a distinct lineage that develops in the thymus, are more immature than iNKT cells that produce IFN-γ and IL-4, and constitute a subset of recent thymic iNKT cell emigrants (23, 24). However, the possibility that mature, uncommitted iNKT cells can respond to activation under the influence of particular cytokines by secreting IL-17 was never addressed.

We investigated whether mature splenic iNKT cells can secrete IL-17 when activated in the same cytokine context that directs Th17 differentiation in naive conventional CD4 T cells. We performed in vitro assays where we assessed the production of IL-17 by unfractionated iNKT cells or by the distinct iNKT cell subsets defined by CD4 and NK1.1 expression stimulated in presence of TGF-β, IL-6, and IL-1β. Surprisingly, we were able to detect IL-17 expression, both intracellular or in the culture supernatants, of all cultured iNKT cells, including the more mature subsets that express NK1.1, CD4, or both. We adoptively transferred these "induced" IL-17–producing iNKT cells to the trachea of naive recipient mice and, even without any further nasal challenge, they were able to induce airway neutrophilia in the lungs. Our results clearly show that IL-17 secretion by iNKT cells is not restricted to the $NK1.1^{-}CD4^{-}$ lineage committed in the thymus, but can also be acquired in the periphery under specific cytokine contexts in which TGF-β, IL-6, and IL-1β are being simultaneously produced. Such peripheral conversion resembles the mechanism that generates induced regulatory T cells (Tregs) in the periphery and endows iNKT cells with functional power.

Materials and Methods

Mice

C57BL/6J (6–8 wk old) mice were bred and maintained under specified pathogen-free conditions at the Instituto Gulbenkian de Ciência. For each experiment, animals were sex and age matched. Experiments were performed according to the guidelines from the National Veterinary Department and Institutional Ethical Comities.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; DN, double-negative; DP, double-positive; GSL-1, glycosphingolipid 1; iNKT, invariant NKT; Nrp-1, neuropilin-1; SP, single-positive; Treg, regulatory T cell.

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In vivo experiments

Mice were anesthetized with ketamine/xylazine, the trachea was surgically exposed, and 50,000 cells were injected. In some experiments, mice received 100 μg neutralizing anti-mouse IL-17 or IFN-γ (clones eBioMM17F3 and R4-6A2, respectively, purchased from eBioscience) 24 h before cell transfer. After 2 d, the mice were sacrificed, the trachea was cannulated, the airways were lavaged three times with 1 ml ice-cold PBS, and the bronchoalveolar lavage (BAL) was collected. The lungs were collected for histological processing and H&E staining.

Flow cytometry and cell sorting

Mouse CD1d tetramers coupled to PE and loaded with PBS57 (an iNKT cell ligand analog to α-galactosylceramide) were supplied by the National Institutes of Health (NIH) Tetramer Facility. Fluorochrome-labeled monoclonal Abs specific to mouse TCRβ (H57-597), NK1.1 (PK136), CD25 (PC61.5), CD4 (RM4-5), CD3 (145-2C11), CCR3 (83103), Gr-1 (RB68C5), MHCII (M5/114.15.2), IL-17 (eBio 17B7), IFN-γ (XMG1.2), and streptavidin were all purchased from eBioscience. Neuropilin-1 (Nrp-1) was detected as described elsewhere (24). For intracellular cytokine detection, cells were incubated for 2 h 30 min with 50 ng/ml 4-α-phorbol 12-mystrate 13-acetate, 500 ng/ml ionomycin, and 10 μg/ml brefeldin A (Sigma). After Ab surface staining, cells were fixed and permeabilized using the Foxp3 staining set (eBioscience), then incubated with unconjugated anti-CD16/32 (clone 2.4G2) rat Ab and with anti-cytokine Abs. Data were acquired in FACSCanto I (Becton Dickinson) and analyzed using FlowJo (Tree Star). For cell sorting, murine iNKT cells were first enriched by magnetic separation, as described elsewhere (25), and samples were sorted on FACSAria I (Becton Dickinson).

In vitro cell cultures

FACS-sorted populations of iNKT or CD4 T cells were cultured in IMDM+ GlutaMAX 5% FBS, 1% penicillin/streptomycin, and 0.01% 2-ME (all from Invitrogen), at 37°C 5% CO_2. For cell stimulation, flat-bottomed 96-well plates were coated for 3 h with anti-CD3 (clone 145-2C11, at 2 μg/ml) and anti-CD28 (clone 37.51, at 5 μg/ml) at 37°C. In some experiments, iNKT cells were stimulated by different concentrations of purified glycosphingolipid 1 (GSL-1; provided by the NIH Tetramer Facility) in the presence of 2×10^4 cells derived from a peritoneal lavage of a naive C57BL/6 mouse as APCs. When indicated, the culture media were supplemented with 1 ng/ml TGF-β (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 10 ng/ml IL-1β (eBioscience), and 10 μg/ml anti–INF-γ (in-house production). At the end of the culture period, supernatants were collected and cells stained for flow cytometry.

IL-17 detection in the culture supernatants

Quantification of IL-17 in culture supernatants was performed in MaxiSorp plates (Nunc) using the mouse IL-17 immunoassay kit (PeproTech), according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using GraphPad Prism 5. The p values were calculated by nonparametric unpaired t test with Welch's correction (Mann–Whitney U test), and p values <0.05 were deemed significant.

Results

Induction of IL-17 production by iNKT cells in presence of IL-1β, IL-6, and TGF-β

It has been reported that IL-17–producing iNKT cells can be identified in several organs (4, 23). We confirmed that within the iNKT population isolated from liver, spleen, or thymus of unmanipulated C57BL/6J mice, a small proportion of cells were positive for IL-17 (Fig. 1A, 1B). In agreement with what has been described, these IL-17$^+$ iNKT cells were mostly CD4$^-$ and NK1.1$^-$ (Fig. 1C).

To investigate whether iNKT cell activation in the presence of cytokines known to favor Th17 polarization could influence iNKT cell fate, we stimulated splenic iNKT cells and naive CD4 T cells isolated from naive mice and purified by FACS with plate-bound anti-CD3 and anti-CD28 in the presence of IL-1β, IL-6, anti–IFN-γ, and TGF-β. T cell activation in the presence of this cytokine mixture has been described as being able to polarize uncommitted CD4 T cells toward Th17 (26–28). We observed that

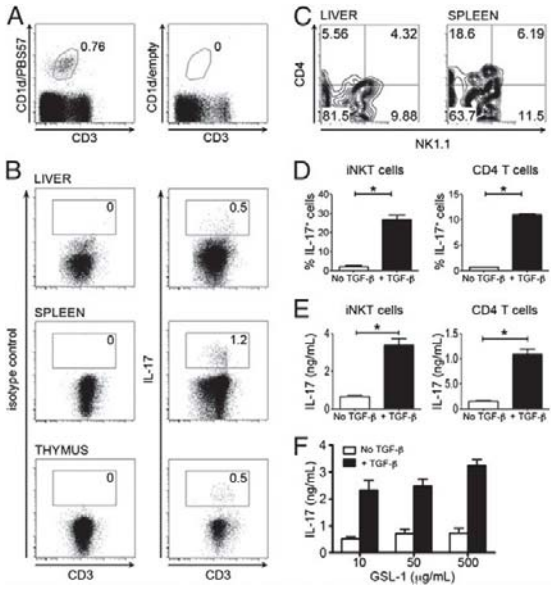

FIGURE 1. Expression of IL-17 by murine iNKT cells is induced in presence of IL-1β, IL-6, and TGF-β. (**A**) Murine iNKT cells from livers, spleens, and thymi of naive C57BL/6J mice (spleen depicted) were indentified as CD1d/PBS57$^+$ CD3$^+$ cells. Unloaded CD1d tetramer control staining to assess background level was also performed. (**B**) Freshly isolated iNKT cells from the liver, spleen, and thymus of unmanipulated C57BL/6J mice were stimulated with PMA/ionomycin in presence of brefeldin A for 3 h, and IL-17–producing cells were identified by flow cytometry. (**C**) CD4 and NK1.1 expression of IL-17$^+$ iNKT cells in the periphery. Data are representative of two independent experiments, each with three mice. (**D**) CD1d/PBS57$^+$CD3$^+$ cells and control CD4$^+$ T cells were purified by FACS and stimulated in triplicate wells by plate-bound anti-CD3 and anti-CD28 in presence of IL-1β, IL-6, anti–IFN-γ, and TGF-β. Negative controls were deprived of exogenous TGF-β addition. After 4 d of culture, cells were further stimulated as described in (B), and the accumulation of IL-17–secreting cells was assessed by flow cytometry. (**E**) IL-17 concentration was assessed by ELISA in the culture supernatants after 4 d of culture. Data are representative of at least three experiments in which culture conditions were set in triplicate. $n = 3$, $*p < 0.05$. (**F**) Splenic iNKT cells negatively enriched through magnetic depletion of CD19$^+$, CD62L$^+$, and CD8$^+$ cells were cultured with peritoneal APCs and stimulated with various concentrations of GSL-1 in the presence of IL-6, IL-1β, and, when indicated, TGF-β. IL-17 concentration was measured in the supernatant after 4 d of culture. Data are presented as means of quadruplicate values ± SEM and are representative of two independent experiments.

iNKT cells responded to the polarizing conditions in a similar way as CD4 T cells, with TGF-β being essential for IL-17 expression (Fig. 1D, 1E). In the end of the cultures, IL-17 could be detected by intracellular staining in ~25% of iNKT cells and 10% of CD4 T cells. Furthermore, IL-17 was present in the culture supernatant of both iNKT and CD4 T cell cultures (Fig. 1E).

Because the in vitro system used to induce IL-17 expression in iNKT cells could involve supraphysiological stimulation, we assessed the capacity of iNKT cells to induce IL-17 when stimulated by a microbial glycolipid presented by APCs. iNKT cells for this assay remained untouched and were enriched only from splenocytes by magnetic depletion of CD19$^+$ CD62L$^+$ and CD8$^+$ cells, to avoid any extra stimulation provided by the tetramer used to identify and isolate these cells by FACS. We then stimulated iNKT cells with different concentrations of GSL-1, a microbial Ag found in *Sphingomonas* spp. (29–31), in the presence of the

cytokine mixture used to polarize Th17 cells. After 4 d of culture, IL-17 concentration in the supernatants was measured (Fig. 1F). In conditions where TGF-β was not added, IL-17 production was relatively low and comparable with the counterpart cultures where iNKT cells were stimulated by plate-bound anti-CD3, independent of the concentration of GSL-1 used for stimulation. In contrast, when TGF-β was added in concert with IL-6 and IL-1β, IL-17 concentration in the supernatant was significantly higher and correlated with the concentration of Ag. These results are consistent with our observations using anti-CD3 stimulation and suggest that iNKT cells might be able to induce IL-17 expression in vivo when stimulated by milder TCR stimuli from microbial origin in the appropriate cytokine environment.

We also evaluated the need for each one of the reagents from the polarization mixture in the induction of IL-17 expression by iNKT cells. For that purpose, we cultured splenic iNKT and naive CD4 T cells as described previously, but in the absence of TGF-β, IL-6, IL-1β, or anti–IFN-γ (Fig. 2A, 2B). In contrast with CD4 T cells, in which IL-17 induction absolutely requires cells to be stimulated in the presence of TGF-β, IL-6, IL-1β, and anti–IFN-γ simultaneously, our results show that maximum IL-17 upregulation in iNKT cells requires both TGF-β and IL-1β (Fig. 2A, 2C), but is independent from exogenous addition of IL-6 and anti–IFN-γ.

These observations indicate that stimulation of iNKT cells in conditions where the cytokines that promote Th17 deviation are present promotes an increased expression of IL-17 by these unconventional T lymphocytes.

All iNKT cell subsets defined by CD4 and NK1.1 expression are able to express IL-17 upon polarization

The identification that IL-17 production by ex vivo–isolated iNKT cells was restricted to a NK1.1$^-$CD4$^-$ subset, together with the identification of the thymic development of such subset, led to the conclusion that IL-17 production was a characteristic of a thymic-derived iNKT cell lineage committed to this particular function (4, 23). However, modulation of the expression of cell-surface molecules after activation of iNKT cells, namely, NK1.1, has been previously reported (32). This prompted us to investigate whether the potential to acquire IL-17 expression was indeed restricted to the NK1.1$^-$CD4$^-$ iNKT cell subset, and whether the CD4 and NK1.1 phenotype remains unchanged after Th17 polarization.

For this aim, we purified by FACS the four iNKT subpopulations defined by the expression of NK1.1 and CD4, or naive CD4 T cells from the spleen of unmanipulated C57BL/6J mice (Fig. 3A), and compared their ability to acquire the expression of IL-17 after activation in conditions that favor Th17 polarization of conventional CD4 T lymphocytes. In the absence of TGF-β, activation of iNKT cells induced high IFN-γ production, but not IL-17, in both NK1.1$^-$CD4$^+$ (CD4 single-positive [SP]) and NK1.1$^+$CD4$^+$ (double-positive [DP]) iNKT cell subsets (Fig. 3B). In contrast, NK1.1$^+$CD4$^-$ (NK1.1SP) and NK1.1$^-$CD4$^-$ (double-negative [DN]) iNKT cell subpopulations exhibited significant expression of IL-17 (6.20 ± 2.01 and 9.20 ± 0.80 cells, respectively) even in the absence of TGF-β (Fig. 3B). These observations might be explained by the expansion of pre-existent IL-17$^+$ iNKT cells known to be contained within the CD4$^-$ iNKT cell population (4, 6, 23).

In conditions in which TGF-β was present, however, all iNKT cell subsets displayed a significant frequency of iNKT cells expressing IL-17 after activation (Fig. 3C) and secreted much higher amounts of this cytokine to the culture supernatant than the CD4 Th17-induced cells (Fig. 3D). These results suggest that, regardless of the cell phenotype in terms of NK1.1 or CD4 expression, iNKT cells retain the potential to acquire IL-17–secreting function after activation under proinflammatory conditions where TGF-β, IL-1β, and eventually IL-6 are present.

We also found that after activation in Th17-polarizing conditions, most iNKT cells downmodulated NK1.1, as it has been widely reported after NKT cell activation (32), and to a lower degree CD4 (Fig. 3E). This phenotypic change brings activated iNKT cells to a phenotype closer to what has been described for the natural IL-17–producing iNKT cells committed in the thymus.

Nrp-1$^-$ iNKT cells are amenable to induce IL-17 expression

It has been shown that iNKT cells that are educated in the thymus to produce IL-17 can be identified within an Nrp-1$^+$ population (24). We therefore investigated whether in vitro induction of IL-17 expression was possible starting from Nrp-1$^-$ iNKT cells purified by FACS (Fig. 4). Nrp-1$^-$ iNKT cells comprise >90% of total splenic iNKT cells and, therefore, could be easily isolated with a high purity degree (Fig. 4A). After 4 d of culture under Th17-promoting conditions, the intracellular expression of IL-17 and IFN-γ were assessed (Fig. 4B, 4C). In the absence of TGF-β, IL-17 expression was undetectable in cultured Nrp-1$^-$ iNKT cells, whereas in the presence of this cytokine, around 3% of the cells expressed IL-17. For comparison, we cultured in parallel Nrp-1$^+$ iNKT cells under the same conditions. As expected, IL-17 expression in Nrp-1$^+$ iNKT cells was drastically higher, with 15% of the cells expressing IL-17 when the culture medium was not supplemented with TGF-β, confirming the existence of a precommitted population of IL-17–secreting iNKT cells (Fig. 4B, 4C). As before, IFN-γ expression was consistently reduced in cultures not supplemented with TGF-β. In summary, these data show that peripheral iNKT cells can induce de novo expression of IL-17 when activated under conditions that promote Th17 deviation in conventional CD4 T cells.

Induced IL-17–producing iNKT cells can drive neutrophilic airways inflammation

Having established that peripheral iNKT cells can be amenable to secrete IL-17 by cytokine polarization, we investigated whether

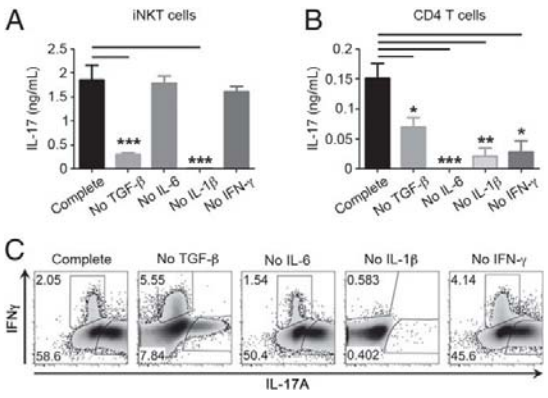

FIGURE 2. TGF-β and IL-1β are essential for IL-17 expression by iNKT cells. iNKT and naive CD4 T cells from the spleen of unmanipulated mice were isolated by FACS as described earlier and stimulated for 4 d by plate-bound anti-CD3 and anti-CD28 in the presence of IL-1β, IL-6, anti–IFN-γ, and TGF-β ("complete" conditions) or in the absence of each one of these reagents. (**A** and **B**) IL-17 concentration was measured in the culture supernatants of iNKT (A) and CD4 T cells (B) at the end of the culture period. (**C**) Intracellular staining for IL-17A and IFN-γ of iNKT cells cultured in different conditions after restimulation with PMA/ionomycin in presence of brefeldin A. This experiment was performed twice with quadruplicates. $n = 4$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.

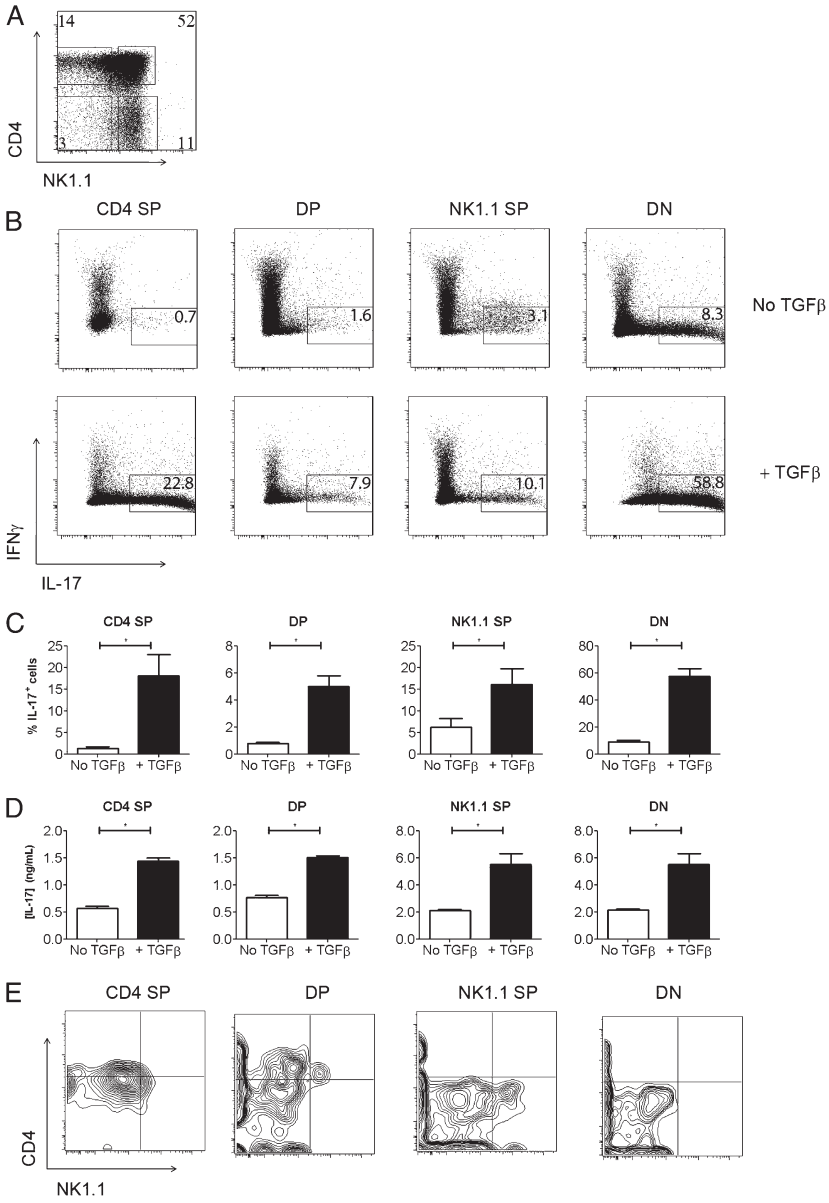

FIGURE 3. Induction of IL-17 expression in the iNKT cell subsets subdivided on the basis of NK1.1 and CD4 expression. (**A**) Splenic iNKT cells from naive C57BL/6J mice were identified as CD1d/PBS57$^+$TCRβ$^+$ cells and further subdivided in subpopulations defined as NK1.1$^-$CD4$^+$ (*CD4 SP*), NK1.1$^+$ CD4$^+$ (*DP*), NK1.1$^+$CD4$^-$ (*NK1.1 SP*), and NK1.1$^-$CD4$^-$ (*DN*). (**B**) The four iNKT cell subsets defined in (A) were purified by FACS, and 50,000 cells of each subpopulation were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-1β, IL-6, and anti–IFN-γ with or without TGF-β. After 4 d of culture, cells were stimulated for 3 h with PMA/ionomycin in the presence of brefeldin A, and flow cytometric analysis of IFN-γ and IL-17 expression by each iNKT cell subset was performed. (**C**) Average frequency of IL-17$^+$ iNKT cells in culture triplicates of the indicated subpopulations. (**D**) Quantification of IL-17 in culture supernatants by ELISA. Bars represent the average of triplicate cultures ± SEM ($p < 0.05$). (**E**) Assessment of NK1.1 and CD4 expression at the end of the culture period. Data are representative of three independent experiments, each with triplicates. *$p < 0.05$.

those induced IL-17$^+$ iNKT cells could maintain their function in vivo, leading to inflammation.

It has been described that IL-17 production can induce inflammatory changes in the airways (6). Thus, we performed intratracheal adoptive cell transfers of 50,000 iNKT cells generated in a 4-d culture period under Th17-polarizing conditions (as described earlier). As control populations, we used an equal number of in vitro–generated Th17 CD4 T cells or activated iNKT cells cultured in medium without TGF-β.

We found that animals transferred with IL-17–producing iNKT cells developed peribronchiolar inflammatory infiltrates, something that was also observed in animals transferred with Th17 cells, but not activated iNKT cells (Fig. 5A). The analysis of the cellular content of the BAL indicated a significant recruitment

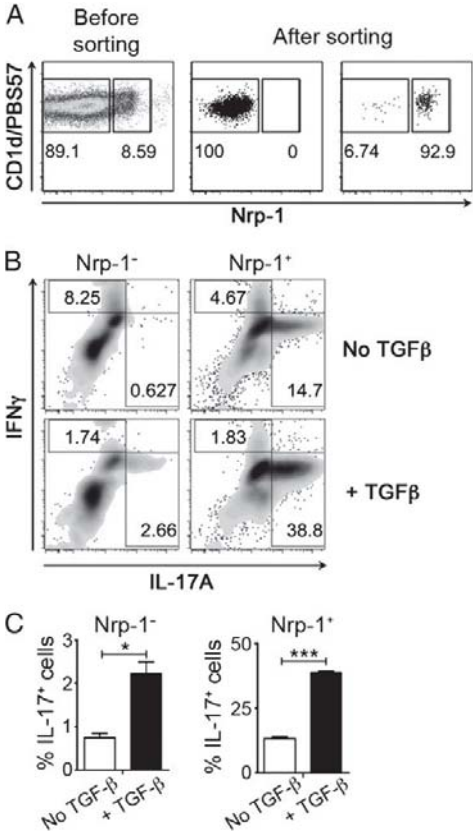

FIGURE 4. Nrp-1$^-$ iNKT cells upregulate IL-17 after culture in the presence of TGF-β, IL-6, and IL-1β. (**A**) iNKT cells were isolated from the spleen of naive C57BL/6 mice and sorted according to the expression of Nrp-1. (**B**) Purified Nrp-1$^-$ and Nrp-1$^+$ iNKT cells were cultured in Th17-polarizing conditions as described earlier, and IL-17 expression was assessed by flow cytometry at day 4. (**C**) Frequency of IL-17$^+$ cells after 4 d of culture of Nrp-1$^-$ and Nrp-1$^+$ iNKT cells assessed by flow cytometry. Data are representative of two experiments, each one with triplicates. $*p < 0.05$, $***p < 0.0001$.

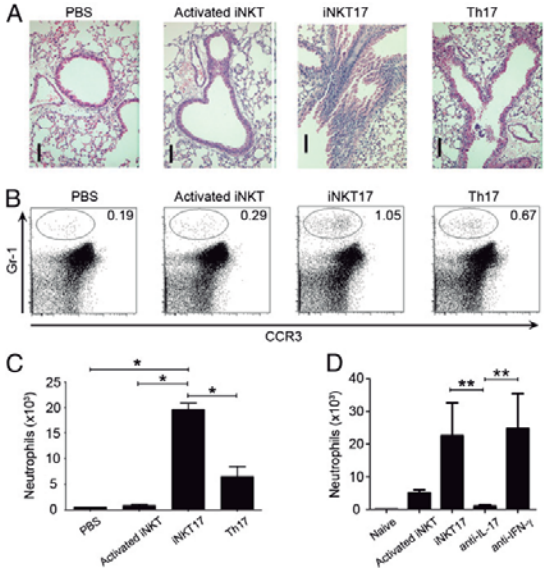

FIGURE 5. The presence of IL-17$^+$ iNKT cells generated by cytokine conversion in the airways is sufficient to induce neutrophilia. IL-17$^+$ iNKT cells were generated in vitro after stimulation of splenic iNKT cells isolated by FACS by plate-bound anti-CD3 and anti-CD28 in the presence of IL-1β, IL-6, anti–IFN-γ, and TGF-β (*iNKT17*). iNKT cells cultured in the absence of TGF-β (*activated iNKT*) and in vitro–generated CD4 Th17 cells (Th17) were used as controls. After 4 d of culture, 50,000 cells or the same volume of 1× PBS was injected in the trachea of naive mice. Animals were sacrificed 2 d postinjection. (**A**) Histology sections of the lungs stained with H&E showing peribronchiolar infiltrates in mice transferred with iNKT17 and Th17 cells. Scale bar, 10 μm. (**B**) Airway neutrophilia was assessed by flow cytometric detection of neutrophils, identified as Gr-1^{hi}CCR3$^{-/+}$MHCII$^-$ cells, in the BAL. (**C**) Absolute quantification of neutrophils in the BAL. A higher percentage (not shown) and absolute number of neutrophils was observed in mice transferred with iNKT17 cells. Data are representative of two independent experiments with three mice per group. (**D**) Absolute numbers of neutrophils in the BAL of mice that have received an adoptive transfer of IL-17$^+$ iNKT cells in the presence or absence of neutralizing anti–IL-17 and anti–IFN-γ mAbs. $*p < 0.05$, $**p < 0.01$.

of neutrophils into the airways in the experimental group that have received IL-17$^+$ iNKT cells that was less pronounced in recipients of Th17 CD4 T cells and undetectable in mice that have received iNKT cells activated without TGF-β (Fig. 5B, 5C). Importantly, the recruitment of neutrophils to the airways was prevented when the mice received an injection of neutralizing anti–IL-17 Ab 24 h before the IL-17$^+$ NKT cell transfer (Fig. 5D). In contrast, neutralization of IFN-γ had no impact in neutrophils' recruitment. These results confirmed that induction of IL-17$^+$ iNKT cells is able to generate a functionally competent population capable of exerting in vivo IL-17–dependent effects comparable with what is observed for Th17 CD4 T cells.

Discussion

In the last 5 y, a growing body of evidence has been raised establishing iNKT cells as one of the several innate sources of IL-17 (6, 11–14, 23). It has been shown that there is an RORγ(t)-dependent NKT cell subset lacking CD4 and NK1.1 expression that commits to IL-17 secretion in the thymus (23). This finding led to the general assumption that the ability of iNKT lymphocytes to secrete IL-17 is restricted to a fixed lineage. In addition, a recent report has demonstrated that IL-17–committed iNKT cells are included in the recent thymic emigrant pool that migrates to the secondary lymphoid organs and is diluted after thymectomy, which suggested that the pool of IL-17–competent iNKT cells present in the lymphoid organs requires continuous thymic output to be maintained (24). Furthermore, because most IL-17–secreting iNKT cells lack NK1.1 expression, it has been postulated that the IL-17 response promoted by NKT cells relies on the activation and expansion of cells from that lineage in the periphery. However, it is well established that NK1.1 expression is profoundly downmodulated after activation, which renders this molecule an unreliable lineage marker in the periphery, in a context of TCR activation.

We have therefore addressed the question of whether the expression of IL-17 by iNKT cells could be an inducible competence when these cells are stimulated under the same conditions promoting the acquisition of a Th17 phenotype by naive conventional CD4 T cells. Indeed, our in vitro studies clearly showed that IL-17 could be readily detected 4 d after initial stimulation of splenic iNKT cells cultured in presence of a cytokine mixture including

TGF-β and IL-1β. The fact that exogenous IL-6 is dispensable for IL-17 expression by iNKT cells is not surprising and has been reported by others (11, 33). However, because splenic iNKT cells have been shown to be able to produce IL-6 in vitro after anti-CD3 and anti-CD28 stimulation (4), the role of this cytokine in induction of de novo IL-17 production remains to be clarified.

Notably, both the frequency of IL-17$^+$ cells and the amount of protein secreted to the culture media were significantly higher in iNKT cells when compared with the CD4 Th17-induced controls, which is in accordance with the fact that NKT cells are proficient cytokine producers early after activation. The emergence of IL-17$^+$ iNKT cells is unlikely to be due to expansion of IL-17–lineage–committed iNKT cells because few IL-17$^+$ cells could be detected in cultures where TGF-β was not added. To exclude this possibility definitely, we adopted two strategies. First, we isolated DP, CD4SP, NK1.1SP, and DN splenic iNKT cell subsets by FACS and assessed induction of IL-17 in each individual population. We observed that IL-17 expression was induced in all iNKT cell subsets cultured under Th17-polarizing conditions, irrespectively of their CD4 and NK1.1 phenotype, with CD4SP and DN populations exhibiting the highest IL-17 expression. Indeed, these two particular subsets, which include the naturally occurring thymus-derived IL-17$^+$ iNKT population, showed 5–10% of IL-17$^+$ iNKT cells in cultures deprived of TGF-β, suggesting that the IL-17$^+$ precursors present have expanded. Of note, despite that exogenous TGF-β has not been provided in this experimental condition, the culture medium might contain traces of this cytokine, and thereby enable some level of TGF-β signal. Nevertheless, the detection of IL-17 in cultures of NK1.1SP and DP subsets, which do not include IL-17–lineage committed cells, clearly demonstrates that it is possible to induce de novo expression of this cytokine in iNKT lymphocytes outside the thymus, under conditions that promote iNKT cell activation and concomitant secretion of TGF-β, IL-6, and IL-1β.

The second strategy to demonstrate that IL-17 might be induced de novo in iNKT cells that had not been committed in the thymus to the NKT-17 lineage consisted of sorting by FACS the iNKT cells lacking the expression of Nrp-1, which are deprived of IL-17–committed cells. Our results show that although Nrp-1$^-$ iNKT cells stimulated in vitro in the presence of TGF-β, IL-6 and IL-1β can indeed upregulate IL-17. However, despite the lower frequency of conversion of Nrp-1$^-$ iNKT cells, compared with Nrp-1$^+$ iNKT cells, one must consider the fact that in vivo Nrp-1$^-$ iNKT cells are at least 10 times more abundant than Nrp-1$^+$ cells. Therefore, when unfractionated splenic iNKT cells are stimulated in conditions that promote Th17 deviation, taking into account that IL-17 expression will be induced or triggered in ~3 and 40% of iNKT cells from Nrp-1$^-$ or Nrp-1$^+$ origin, respectively, it is reasonable to assume that >40% of the IL-17$^+$ iNKT cells are generated from the Nrp-1$^-$ precursors. Thus, the contribution of the Nrp-1$^-$ iNKT cell pool for the total number of IL-17$^+$ cells is similar to the input from the Nrp-1$^+$ iNKT cell subset, suggesting that peripheral induction of IL-17 in iNKT cells may be relevant in physiological proinflammatory conditions.

Our results also show that induced IL-17$^+$ iNKT cells have almost completely downregulated NK1.1 expression from the surface and, to a lesser extent, have downmodulated CD4. However, it is difficult to accurately correlate the expression of IL-17 to NK1.1 and CD4 because the cells were stimulated in presence of a Golgi apparatus and endoplasmic reticulum protein transport inhibitor. Nevertheless, these observations indicate that in vivo NK1.1 expression is not useful to distinguish IL-17$^+$ NKT cells of the thymic lineage from their peripherally generated counterparts. The expression of Nrp-1 by thymus-derived IL-17$^+$ NKT cells (24) might represent a better strategy to circumvent this limitation.

In Fig. 3B, we show the expression of IFN-γ and IL-17A after culture under Th17-polarizing conditions in iNKT cell subsets defined by NK1.1 and CD4 expression. Irrespective of the presence of TGF-β, IFN-γ$^+$ cells are always detected in every subset. Most of these IFN-γ$^+$ cells are IL-17A$^-$, but we cannot exclude the presence of a minor population of cells that coexpress both IL-17A and IFN-γ. The existence of a double-producer population of IL-17A and IFN-γ has been documented for human CD4 Th17 cells (34–36) and, at least in these cells, correlates with a Th17 subset that coexpresses both CCR6 and CXCR3 chemokine receptors. This subset is distinct from Th17 cells that coexpress CCR6 together with CCR4, which, in turn, secrete IL-17 and IL-22. This dichotomy derives in CD4 Th17 cells from distinct activation contexts, in particular, in response to different types of pathogens: for instance, whereas memory Th17 cells specific for *C. albicans* are primarily found within the CCR6$^+$CCR4$^+$ subset, Th17 cells specific for *M. tuberculosis* are mainly CCR6$^+$CXCR3$^+$. This reflects a fine-tuned coordination between the differentiation of the appropriate effector functions in response to a given stimulus and the migratory capacity of the effector cells. In the case of iNKT cells that have acquired IL-17 in vitro, it is possible that a similar association between effector molecules and distinct migratory potentials might exist. Indeed, studies of human iNKT cells had already established a correlation between the type of chemokine receptors expressed by Vα24Vβ11 T cells and the expression of Th1 and Th2 cytokines, with IL-4 and IFN-γ being preferentially expressed by iNKT subsets expressing CCR4 and CXCR3, respectively (37–39). Therefore, when we transferred such heterogeneous population to the trachea of mice, in addition to the clear effect seen in the neutrophil recruitment to the lungs in result of the IL-17 production, other cell populations might as well be affected by other cytokines being produced.

The fact that IL-17$^+$ NKT cells might have a central and peripheral origin resembles the scenario observed for Tregs, a specialized population of CD4 T lymphocytes fundamental for the maintenance of immunological self-tolerance (40, 41). Tregs can be generated in the thymus from CD4$^+$CD8$^+$ DP thymocytes that undergo positive selection and acquire the expression of the transcription factor Foxp3, or in the periphery, from naive CD4 T cell precursors that upregulate Foxp3 in response to activation in presence of locally produced TGF-β (42–45). The first are generally called natural Tregs and the latter induced Tregs, and both are capable of exerting suppression of other cells' functions by similar mechanisms. Our results indicate that a similar dual origin for IL-17$^+$ iNKT cells might coexist physiologically. We therefore propose that NKT cells committed to IL-17 secretion in the thymus should be called "natural NKT17" cells, in contrast with the "induced NKT17 cells" generated in the periphery.

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Disclosures

The authors have no financial conflicts of interest.

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Review Article

Regulatory T Cells and the Control of the Allergic Response

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The study of immune regulation and tolerance has been traditionally associated with self/nonself-discrimination. However, the finding that dominant tolerance, a model that puts in evidence the active role of regulatory T cells, can develop to nonself-antigens suggests that the imposition of tolerance can be context dependent. This paper reviews the emerging field of acquired immune tolerance to non-self antigens, with an emphasis on the different subsets of induced regulatory T cells that appear to specialize in specific functional niches. Such regulatory mechanisms are important in preventing the onset of allergic diseases in healthy individuals. In addition, it may be possible to take advantage of these immune regulatory mechanisms for the induction of tolerance in cases where pathological immune responses are generated to allergens occurring in nature, but also to other immunogens such as biological drugs developed for medical therapies.

1. Introduction

For many decades the self/nonself-discrimination by the immune system was assumed to be a consequence of clonal selection of effector T cells. Compelling evidence has, however, imposed a revised view of self/nonself-discrimination: dominant regulatory mechanisms, where regulatory T (Treg) cells play a central role, are essential for maintenance of self-tolerance [1]. But recently it is becoming apparent that the importance of dominant regulation goes beyond the discrimination of self and nonself: it also discriminates between harmful and innocuous. In fact, cellular mechanisms, as detailed below, persistently patrol the organism preventing the onset of inflammation, namely, allergic inflammation. The biological significance of this active tolerance-imposing mechanism is well demonstrated by the severity of the allergic and autoimmune syndrome that arises in individuals that lack these ability to tolerate self- and harmless antigens.

Indeed, the organism is constantly exposed to non-pathogenic antigens that, in healthy individuals, are tolerated. It is, however, common (and becoming increasingly frequent) that an overzealous immune system will activate and develop effector responses to such harmless antigens developing allergy and other inflammatory diseases. Over the last decades allergic diseases, including allergic asthma, atopic dermatitis, and food allergy, have become a major health problem in developed countries [2]. Despite the advances in the understanding of the pathophysiology of allergy and in its clinical management, allergic pathology remains a significant burden on the quality of life and economy of western society. Several strategies have been devised to overcome the pathological immune response by inducing immune tolerance. This paper reviews the impact of dominant regulatory mechanisms in the maintenance of tolerance to foreign antigens, including allergens.

A major cellular mechanism in maintaining immune tolerance is the population of natural (or thymic-derived) Foxp3$^+$ Treg cells [3, 4]. Indeed these have been clearly implicated as potent inducers of a nonresponsive state in several immune-mediated pathologies like autoimmunity, transplantation, graft-versus-host disease, and allergy [5–9]. It has been shown, in allergy, that regulatory T cells can be transferred conferring specific tolerance to subsequent challenges with the allergen [10, 11]. In addition, depletion of the regulatory T cells can have a detrimental effect in allergic airway hyperreactivity [12]. Importantly Foxp3 deficiency, in

mice and human beings, leads to a severe immune disregulation syndrome characterized by allergic and autoimmune manifestations that are rapidly fatal [13]. In addition to the important role of natural Foxp3$^+$ Treg cells (nTreg) in preventing autoimmunity, it has become established that Foxp3 expression can be peripherally induced following T-cell activation in presence of TGF-β [14]. These peripherally induced Treg cells (iTreg) are believed to be important for tolerance induction to nonselfantigens, including allergens [14].

2. Induction of Regulatory T Cells

The study of peripheral induction of Treg cells was greatly facilitated with the use of *Rag*-insufficient TCR-transgenic mice, with the TCR specific for a nonselfantigen. In these mice nTregs cannot be formed in the thymus due to the absence of a selecting thymic antigen. In 2003 it was shown that conventional T cells can be converted into iTreg *in vitro* when activated in presence of TGF-β [15]. In addition those iTreg cells were fully capable of controlling airway hyperreactivity (AHR) in previously sensitized mice [15–19]. It was subsequently found that reducing or blocking the available amount of TGF-β exacerbates AHR [20, 21], while the local delivery of this cytokine or adoptive transfer of T cells engineered to express latent TGF-β rescue mice from antigen sensitization and therefore prevent AHR [22, 23]. Interestingly, suboptimal TCR signaling together with TGF-β greatly enhances iTreg conversion [24], which is in agreement with *in vivo* data showing that repeated low doses of allergen exposure promotes the emergence of Foxp3$^+$ iTregs expressing TGF-β on the membrane [25]. Under suboptimal TCR stimulation, which can be obtained by using a low dose of plate bound anti-CD3 or DCs pulsed with a low dose of agonist peptide or with downmodulation of the TCR with nondepleting anti-CD4, iTreg conversion is promoted in the absence of exogenous TGF-β [26]. Under those conditions Foxp3 expression still requires TGF-β, but the T cells can produce TGF-β and benefit from the presence of this cytokine for conversion to Treg [26].

In addition to the importance of TGF-β for iTreg conversion, some studies showed that TGF-β can directly inhibit GATA3 expression thus impairing Th2 differentiation [27–29]. Because the Th2 response is impaired, the production of IL-4 is diminished, and this has a direct impact on B-cell class switch preventing IgE and favoring IgA production [30].

It is also becoming apparent that the environment influences the outcome of T-cell activation and the decision to induce Foxp3 and regulatory properties. Several reports have shown that the mucosal surfaces have a role in establishing an iTreg population: alveolar epithelial cells have been reported to participate in iTreg induction in a mechanism dependent of MHC class II expression and TGF-β [31]. Both alveolar and gut epithelia have been shown to depend on retinoic acid together with TGF-β to induce tolerance [32, 33]. It was also found that retinoic acid in the presence of TGF-β impaired STAT6 binding to the Foxp3 promoter therefore enhancing histone acetylation and reverting the repressive effect of IL-4 on the Foxp3 promoter [34].

Despite the critical role of TGF-β in iTreg induction and henceforth tolerance, this cytokine can also have some adverse effects since it is instrumental in the differentiation of Th17 and Th9 together with IL-6 and IL-4, respectively [35, 36]. Note that although Treg cells can prevent allergic autoimmune encephalomyelitis (EAE), mice with T cells with a dominant negative receptor for TGF-β1 do not develop EAE as Th17 cells are not induced [37]. Moreover, TGF-β has also been implicated in tissue remodeling, by induction of collagen expression in fibroblasts, as well as goblet cell proliferation and mucus production [38].

3. Regulatory T Cells and IL-10

Although TGF-β is the major known driver of iTreg differentiation, IL-10 has been shown to be another key player that has been vastly described in protection from allergic diseases [39].

Studies with bee venom-specific immunotherapy have shown that tolerance to the allergen can be induced in a process that is IL-10 mediated [40]. In addition, respiratory exposure tolerance induction to OVA relied on antigen specific CD4$^+$ regulatory cells that produced IL-10 [41]. Tolerance was transferrable and abrogated when IL-10 or ICOS ligand was blocked. Interestingly, those regulatory cells shared some features with effector Th2 cells: both populations expressed IL-4 and IL-10 although in different amounts. While the regulatory cells primarily release IL-10, the effectors rely on IL-4 as the main cytokine. It has been suggested that different types of effector cells, including Th2, produce IL-10 at the end of the immune response in a mechanism that is important in limiting their inflammatory behavior [42]. IL-10 producing T cells has been described able to control the late response in allergic asthma by reducing neutrophilia [43]. It has been suggested that Foxp3-negative IL-10 producing T cells can be induced following activation in presence of IL-10 and constitute a population of regulatory cells different from Foxp3$^+$ Tregs that are named TR1 [44, 45]. TR1 cells have been identified in mice and humans, and there are currently clinical trials [9, 46]. There are several other lines of evidence demonstrating the crucial role of IL-10 in the prevention of airway inflammation: IL-10-deficient mice have an exacerbated allergic airways response with high levels of proinflammatory cytokines like IL-5 and IFN-γ in the BAL [47]. Furthermore, intranasal administration of rmIL-10, concurrently with OVA, inhibited both airway neutrophilia and eosinophilia [48]. It was also shown that allergen-specific CD4$^+$CD25$^+$ Tregs can suppress allergic airway disease *in vivo* through an IL-10-dependent mechanism [18]. In this study, adoptive transfer of Treg cells reduced AHR, Th2 and eosinophil recruitment into the airways, and secretion of Th2-type cytokines. The effect was IL-10 mediated, since neutralizing anti-IL-10R abrogated suppression. In addition, these effects were independent of IL-10 production by the CD4$^+$CD25$^+$ regulatory cells themselves [18].

Unlike TGF-β, IL-10 does not directly influence B-cell class switch [49]. However, it is possible that indirectly, by inhibiting the inflammatory response, IL-10 shapes the

humoral outcome. Indeed, it was proposed that IL-10 may favor the ratio of IgG4/IgE ratio [50]. In fact immunotherapy studies show that Th2 responses can be suppressed by IL-10 secreting regulatory cells accompanied by an increase of circulating IgG4 [51, 52].

4. Different Subsets of Regulatory T Cells

Foxp3$^+$ Treg cells, despite an apparent phenotypic uniformity and immunosuppressive function, can have different subtypes with distinct genetic signatures. The first major division was identified between nTreg and iTreg, where the first are enriched in Helios, a transcription factor that is primarily expressed in T-lineage cells and early precursors [53, 54]. While nTreg cells have epigenetic mechanisms that stabilize Foxp3 expression allowing them to be a stable differentiated cell lineage, TGF-β induced Tregs lack those mechanisms having incomplete demethylation [55]. Therefore, although iTreg cells have high levels of Foxp3, the expression of Foxp3 is less stable [55–57]. In addition, conserved noncoding DNA sequence (CNS) elements at the Foxp3 locus encode information defining the size, composition, and stability of the Treg cell population [58]. CNS3, which binds c-Rel, has a drastic effect on the frequency of Treg cells generated in the thymus. Contrary to CNS3, CNS1 has no effect on thymic generation of Treg cells but is essential for induction of iTregs [58]. CNS1 contains a TGF-β-NFAT response element, so these results could represent the requirement of TGF-β and NFAT for Treg induction in the periphery [58–60]. Although CNS2-deficient T cells can acquire Foxp3 expression, they fail to maintain Foxp3 expression on their progeny due to the failure on recruitment of Foxp3-Runx1-Cbf-β complexes to CNS2 after demethylation of the CNS2 CpG island [58, 61]. Interestingly CNS1 deficient mice had no lymphoproliferative disorder. However, it can be argued that these animals kept in clean facilities have a minimal exposure to foreign antigens and thus nTreg may be sufficient to maintain homeostasis in such conditions. In effector T cells, GATA-3 is a hallmark of the Th2 cells, but Treg cells can also express GATA-3, that binds both to the Th2 cell locus and to the CNS2 of Foxp3 locus [62]. In fact, there is a dramatic increase of GATA-3 binding to CNS2 compared to conventional T cells, suggesting that GATA-3 regulates CNS2 activity in Treg cells [62].

There is strong evidence that the CCR7-dependent continuous migration of DC from the lung to its draining LNs is required for the transport of inhaled Ag and thereby for the proper composition of APCs in the LN. These processes are essential to induce peripheral tolerance of T cells [63]. The costimulation with ICOS, crucial for regulatory phenotype polarization in allergy [64], promotes the downregulation of CCR7 and CD62L after activation, leading to a reduced return of activated CD4 T cells to the lymph nodes and a more efficient entry into the lungs [65]. Regulatory T cells express CCR4 and CD103 induced by antigen-driven activation in the lymph nodes. In addition, the accumulation of Tregs in the skin and lung airways is impaired in the absence of CCR4 expression [66]. Mice without CCR4 in the Treg compartment develop lymphocytic infiltration and severe inflammatory disease in the skin and lungs [66]. Some studies suggest that CCR4 has a prominent role in effector Th2 homing [67]. Despite their differences it seems both regulatory and effector T cells share the response to homing factors [68, 69].

But GATA-3 is not the only transcription factor characteristic of effector T-cell responses that can be expressed by Foxp3$^+$ Treg cells. Under the influence of IFN-γ, Foxp3$^+$ Treg cells can express the Th1-defining transcription factor T-bet [70]. T-bet expression by Foxp3$^+$ Treg cells induces the expression of the chemokine receptor CXCR3, necessary for these Treg cells to accumulate at the site of type 1 inflammation. T-bet expression was thus required for the homeostasis and function of Treg cells during type-1 inflammation [70].

It is likely that the regulation of different types of immune response requires the participation of specialized subsets of regulatory cells. This way, iTreg cells induced in an environment favorable to Th1 or Th2 type of immune responses require the appropriate chemokine receptors to give them access to the same locations as effector T cells (Figure 1).

Th17 cells that have been implicated in autoimmunity and allergy share with iTreg cells the need for TGF-β to differentiate [71]. The decision of antigen-stimulated cells to differentiate into either Th17 or iTreg depends on the cytokine balance of IL-6, IL-21, and IL-23 that relieve Foxp3-mediated inhibition of RORγt [72]. These results indicate that Foxp3 and RORγt are transcription factors that antagonize each other in the lineage differentiation.

Another subset of T cells, the follicular T helper cells (Tfh), is mostly spatially confined to secondary lymphoid organs, more precisely to the B-cell follicles [73]. Tfh cells express high levels of the transcription factor Bcl-6, that impairs the expression and function of other transcription factors specific for other CD4 subsets: Tbet, GATA3, and RORγt, thereby regulating cytokine production by Tfh cells [74, 75]. Tfh cells differentiate under the influence of ICOS:ICOSL and IL-21 but independently of any other cytokine [76]. In addition, the characteristic anatomical distribution of Tfh cells is dependent of CXCR5 that endows access to the B-cell follicle [73, 77, 78]. We and others have recently found that also this subset of effector T cells has a specialized regulatory counterpart [69, 79, 80]. It was found that Foxp3$^+$ Treg cells can be found within the B-cell follicle [81], sharing many characteristics of Tfh and Treg cells [69, 79, 80]. Importantly, Bcl-6 can be coexpressed with Foxp3 as it seems Foxp3 expression is not inhibited by Bcl-6. These follicular regulatory T cells (Tfr) are immunesuppressive and can control de magnitude of the germinal center response [69, 79, 80]. In addition, they exhibit a CTLA4^{hi}GITR^{hi}IL-10^{hi} phenotype that is the characteristic of activated Tregs [69, 79, 80]. However, the Tfr origin is quite distinct from the other induced Treg cells previously described. Tfr cells do not derive from the commitment of conventional CD4 T cells, but result from acquisition of "follicular" characteristics (viz. Bcl-6 expression) by natural Foxp3$^+$ Treg cells [69, 79, 80]. In fact sorted Tfh cells

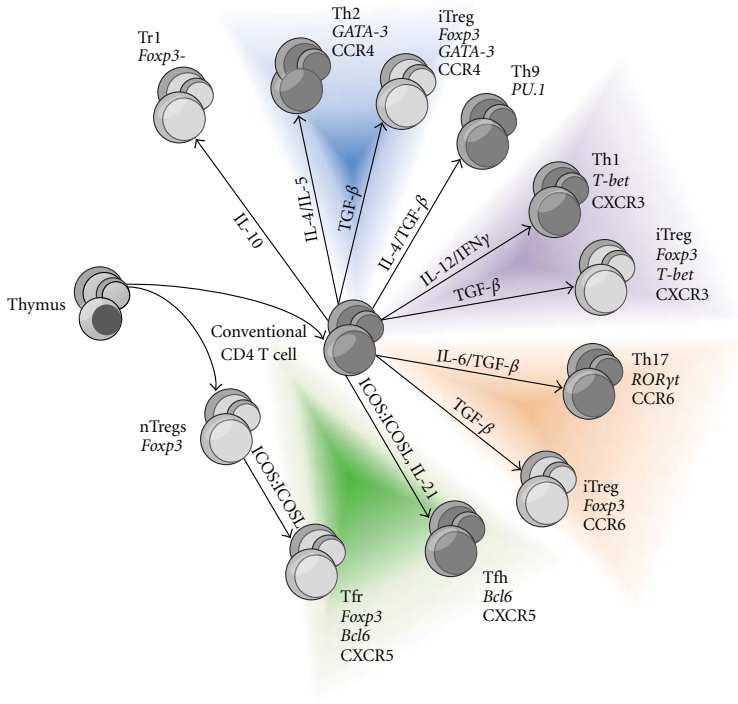

Figure 1: Functional specialization of effector and regulatory T cells. Different types of immune responses carry different cytokine microenvironments that can influence both effector and regulatory T-cell differentiation. In the same way effector T cells when activated in specific cytokine environment acquire specialized functions, induced regulatory cells (iTreg) can also activate the expression of different transcription factors (italics) that endow them access to different anatomic compartments on the basis of the chemokine receptors they express. Follicular regulatory cells (Tfr) represent an exception among peripherally induced Foxp3$^+$ cells, as they are derived from natural regulatory cells (nTreg) that acquire Bcl-6 expression, rather than from conventional CD4 T cells.

exposed to optimal conditions to induce Foxp3 expression in conventional T cells (including TGF-β) resist conversion to Tfr [79]. Given the importance of the germinal center response for allergy, it is likely that Tfr cells can play an important role in regulating IgE production.

Besides conventional T cells, also natural killer T (NKT) cells are important players in defining the outcome of immune responses. Notably, invariant NKT (iNKT) cells were found able to help B-cell differentiation, germinal-center formation, affinity maturation, and immunoglobulin response that was uniquely dependent on iNKT cell-derived IL-21, although the GCs maintain a small size throughout the reaction [82, 83]. This contribution of iNKT cells for humoral responses can be added to their ability to contribute to allergic airways diseases by producing IL-4 and IL-13 [84, 85], or IL-17 [86, 87]. But iNKT cells can also have a regulatory role, namely, in preventing EAE following administration of its TCR agonist [88, 89]. We and others recently described that activation of murine or human iNKT cells in presence of TGF-β induces Foxp3 expression and acquisition of suppressive function [88, 90].

5. Influencing Regulatory T Cells in Allergy

The understanding of the mechanisms involved in regulatory T-cells generation and function may lead to novel strategies to restore immune tolerance where it has been lost. As TGF-β and IL-10 play a crucial role in tolerance induction, several studies on immune tolerance induction took advantage of environments rich in those anti-inflammatory cytokines. To our advantage the mucosa itself is an anatomical location rich in these immune mediators [91].

Airborne antigens can be transferred from the mother to the newborn through milk [92]. Breastfeeding-induced tolerance was found to be mediated by induced Foxp3$^+$ Treg cells and dependent on TGF-β [92]. It has been proposed that metallomatrix proteases, derived from commensal bacteria in the gut, can facilitate the conversion of latent TGF-β to its active form, thus favoring iTreg differentiation [93]. In addition, CD103$^+$ dendritic cells in the mucosa-draining lymph nodes have been shown effective in promoting conversion of iTregs in the gut, mediated by TGF-β and the synthesis of retinoic acid, a powerful inducer of Foxp3 expression [32, 94, 95]. Furthermore, vitamin D receptor

deficient mice were associated with a reduction in tolerogenic $CD103^+$ dendritic cells favoring the development of effector type T cells [96]. Vitamin D3 can be used to induce human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into regulatory cells that produced only IL-10, but no IL-5 and IFN-γ, and furthermore retain strong proliferative capacity [97]. Several other studies put vitamin D3 in relevance as acting directly on T cells to induce IL-10^+ regulatory cells and also influencing levels of TGF-β [98–100]. These data suggest that the mucosa, in particular the gut, has several mechanisms that can favor immune tolerance. Sublingual immunotherapy (SLIT) and oral immunotherapy (OIT) are becoming more relevant as effective tolerance-inducing strategies to treat inhalant as well as food allergies [101].

Allergen specific immunotherapy (SIT) which comprehends SLIT, OIT, and subcutaneous immunotherapy (SCIT) has been in clinical use for around 100 years [102] and consists on the administration of increasing doses of an allergen [103]. It has been shown that both $Foxp3^+$ and IL-10 positive regulatory T cells can be induced during the course of SIT protocols [104, 105]. Furthermore, allergen-specific TR1 cells, in healthy individuals, have been suggested to play a key role in preventing pathologic responses [52, 102, 106]. While the presence of IL-10 leads B cells to produce IgG4 in detriment of IgE [107, 108], TGF-β drives B cells to switch to IgA production [106]. Another approach to direct the organism towards a tolerant state arises from the results that suggest that reduced TCR stimulation favors the induction of a regulatory phenotype on the T cells [26, 109, 110]. Blockade of molecules involved in the immune synapse has been suggested as an approach to achieve suboptimal TCR activation [26, 110]. Blockade of CD4 was shown a robust approach to achieve Treg-mediated dominant tolerance in transplantation [111–113]. We recently showed that a nondepleting anti-CD4 monoclonal antibody can induce in mice robust, antigen-specific tolerance to house dust mite, even in presensitized animals [16]. In addition, a similar strategy was effective to prevent peanut-induced anaphylaxis in mice [114]. Costimulation blockade was also shown effective in preventing allergic sensitization in mice [115]. Based on previous studies of tolerance induction to alloantigens following costimulation blockade, it is likely the mechanism also relies on Treg cells [116, 117]. Regarding the different modalities for costimulation blockade, on one hand CTLA4Ig was shown able to greatly reduce the secretion of IL-4 but not enough to impair Th2 response [118]. On the other hand, treatment with OX40L-blocking mAbs inhibited to some extent allergic immune responses induced by TSLP in the lung and skin, preventing Th2 inflammatory cell infiltration, cytokine secretion, and IgE production in mice and nonhuman primate models of asthma [119].

6. Final Remarks

The realization that active regulatory mechanisms, such as the ones mediated by Treg cells, can prevent pathological immune responses to harmless antigens is changing the way immunotherapy is perceived. In very diverse fields of immunology, ranging from cancer immunotherapy to autoimmunity and allergy, regulatory mechanisms need to be considered when therapeutic interventions are designed to boost or dampen the immune response. The realization that different subsets of regulatory T cells exist may offer the possibility to fine tune such interventions in order to achieve optimal therapeutic benefit with limited immunosuppressive consequences in unrelated immune responses.

At a time when therapeutic interventions rely increasingly on potentially immunogenic drugs, such as recombinant proteins to correct genetic diseases or monoclonal antibodies, where even the human antibodies can be immunogenic due to their unique idiotypes [120, 121], the issue of tolerance induction to nonselfantigens will not be restricted to allergy and transplantation, but a growing concern for drug efficacy.

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T Cell Apoptosis and Induction of Foxp3$^+$ Regulatory T Cells Underlie the Therapeutic Efficacy of CD4 Blockade in Experimental Autoimmune Encephalomyelitis

Joana Duarte,[*,†] Nadège Carrié,[‡,§] Vanessa G. Oliveira,[*,†] Catarina Almeida,[*,†] Ana Agua-Doce,[*,†] Lénia Rodrigues,[*,†] J. Pedro Simas,[*,†] Lennart T. Mars,[‡,§] and Luis Graca[*,†]

The pathogenesis of multiple sclerosis requires the participation of effector neuroantigen-specific T cells. Thus, T cell targeting has been proposed as a promising therapeutic strategy. However, the mechanism underlying effective disease prevention following T cell targeting remains incompletely known. We found, using several TCR-transgenic strains, that CD4 blockade is effective in preventing experimental autoimmune encephalopathy and in treating mice after the disease onset. The mechanism does not rely on direct T cell depletion, but the anti-CD4 mAb prevents the proliferation of naive neuroantigen-specific T cells, as well as acquisition of effector Th1 and Th17 phenotypes. Simultaneously, the mAb favors peripheral conversion of Foxp3$^+$ regulatory T cells. Pre-existing effector cells, or neuroantigen-specific cells that undergo cell division despite the presence of anti-CD4, are committed to apoptosis. Therefore, protection from experimental autoimmune encephalopathy relies on a combination of dominant mechanisms grounded on regulatory T cell induction and recessive mechanisms based on apoptosis of neuropathogenic cells. We anticipate that the same mechanisms may be implicated in other T cell-mediated autoimmune diseases that can be treated or prevented with Abs targeting T cell molecules, such as CD4 or CD3. ***The Journal of Immunology*****, 2012, 189: 1680–1688.**

Multiple sclerosis (MS) is an inflammatory disease of the CNS leading to progressive demyelination that is the leading cause of neurologic disability among young adults (1, 2). Genetic risk factors indicate that the heritable risk of developing MS is associated with allelic variants of predominantly immune-related genes (3–7). The strongest association is conferred by the human leukocyte/Ag locus (8). The inflammatory response present within the demyelinating lesions mainly comprises CD4 and CD8 T cells as well as macrophages (9). The commonly held view is that this response is autoreactive, targeting CNS-derived Ags (10). The pathogenicity of this response is modeled in experimental autoimmune encephalomyelitis (EAE), a paralytic demyelinating disease that is induced in susceptible animals after immunization with myelin Ags, such as myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) (11). By using these models, myelin-reactive B cells and CD8$^+$ and CD4$^+$ T cells have been identified as effector cells able to drive CNS demyelination. Furthermore, functionally distinct CD4 T cell subsets (Th1 and Th17) are now recognized to cause CNS tissue damage with distinct pathological features (10). Their pathogenicity is essentially mediated by the production of GM-CSF (12–14). Conversely, regulatory T cells (Treg) have been shown to limit the pathology or to prevent its onset (15–17).

Given the important role of T cells in the pathogenesis of MS, several therapeutic strategies have been proposed that rely on T cell targeting, namely T cell depletion, achieved with alemtuzumab, an anti-CD52 mAb (18), but also strategies that block α_4 integrin function (19–22), as well as T cell retention by FTY720 (23, 24). CD4 blockade, however, with nondepleting mAbs (that do not lead to direct cell lysis) offer the opportunity to specifically target Ag-specific cells engaged in T cell activation at the time of mAb treatment, rather than having a nonspecific effect. Other mAbs targeting molecules involved in T cell activation (such as CD3 or CD154) can also lead to long-term immune tolerance in preclinical models (25–28). Of note, anti-CD3 therapy generated promising results in phase I/II clinical trials, delaying onset of type I diabetes and treating psoriatic arthritis (29–31).

We have taken interest in nondepleting anti-CD4 mAb because of its established capacity to induce T cell tolerance without imposing systemic immune-suppression (32–41). The efficacy of nondepleting anti-CD4 in transplantation stems from its ability to impose a state of persistent T cell unresponsiveness, even when the mAbs have long disappeared from the circulation, that can spread to naive T cells through what was termed "infectious tolerance" (42, 43).

Promising results in EAE following anti-CD4 treatment (32, 44–46) justified some of the earliest clinical trials of mAbs in MS in the early 1990s (47). However, the immaturity of the field at that time led to discouraging results, namely due to the immunogenicity of the mAb (it was not humanized) and to adverse

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Abbreviations used in this article: EAE, experimental autoimmune encephalopathy; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; MuHV, murid herpesvirus; TR$^-$, TCR-transgenic Rag1$^{-/-}$; Treg, regulatory T cell.

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effects due to T cell depletion (48).The mechanisms underlying the putative tolerogenic effect of anti-CD4 treatment in neuroinflammation were never fully characterized.

In this study, we evaluate the mechanisms underlying the protective effect of CD4 blockade in EAE. Using models of spontaneous and active EAE, we show that a nondepleting isotype of anti-CD4 mAb can prevent and treat EAE without leading to CD4 T cell depletion. Treated mice remained immunocompetent against viral infection.

We found that the underlying mechanism of CD4 blockade is distinct in naive and preactivated cells. Whereas CD4 blockade on activated cells favored their commitment to apoptosis, CD4 blockade during the priming of naive cells halted proliferation and effector differentiation and promoted the emergence of $Foxp3^+$ Tregs.

It is a combination of halting the effector function of preactivated cells together with the recruitment of naive cells into the regulatory pool that allows CD4 blockade to be effective in TCR-transgenic mice, even after the onset of the disease. As such, our data provide insight into the cellular mechanisms that are deployed to achieve EAE protection with putative tolerogenic mAbs.

Materials and Methods

Animals

C57BL/6 (B6; H-2^b), MBP-specific TCR-transgenic Rag1$^{-/-}$ (TR^-) mice (H-2^u) (49), and 2D2 MOG-specific TCR-transgenic mice (H-2^b) (50) were bred and maintained under specific pathogen-free conditions and used sex matched between 8 and 10 wk age. All experimental protocols were approved by the Local Ethics Committee and are in compliance with European Union guidelines.

EAE induction

For active EAE, B6 mice were s.c. immunized with 100 μg $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) (Biopolymers Laboratory, Harvard Medical School) emulsified in CFA solution (4 mg/ml mycobacteria in IFA). On the day of immunization and 2 d after, mice received 200 ng pertussis toxin (List Biological Laboratories) in 100 μl PBS i.v. For 2D2 Th1 transfer, purified $CD4^+$ T cells from 2D2 mice at 0.5×10^6 cells/ml were stimulated in vitro with 20 μg/ml NF-$M_{15–35}$ (RRVTETRSSFSRVSGSPSSGF) peptide in the presence of IL-12 (20 ng/ml), IL-2 (1 ng/ml), and irradiated syngeneic splenocytes ($5–10 \times 10^6$). On day 6, viable cells were restimulated with APCs and 20 μg/ml NF-$M_{15–35}$ in the presence of IL-12 (20 ng/ml) and IL-2 (1 ng/ml). On day 9, 14×10^6 Th1 cells were injected into lightly irradiated (300 rads) syngeneic recipients. Disease severity was scored daily on a five-point scale: 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, quadriplegia; 5, moribund.

Abs

Nondepleting anti-CD4 (YTS177) and isotype control (YKIX302) mAbs were produced in our laboratory using Integra CL1000 flasks (IBS Integra Biosciences, Chur, Switzerland), purified by 50% ammonium sulfate precipitation, dialyzed against PBS, and purity was checked by native and SDS gel electrophoresis. Hybridomas were generously provided by Prof. Herman Waldmann (Oxford, U.K.).

Preparation of CNS mononuclear cells

Mice were perfused through the left cardiac ventricle with cold PBS. The brain and spinal cord were dissected. CNS tissue was cut into pieces and digested with collagenase type VIII (0.2 mg/ml; Sigma-Aldrich) in HBSS at 37°C for 30 min. Mononuclear cells were isolated by passing the tissue through a 70-μm cell strainer, followed by a 30% Percoll (Sigma-Aldrich) gradient and 20 min centrifugation at 2500 rpm. Mononuclear cells were recovered from the pellet, resuspended, and used for further analysis.

Flow cytometry

Single-cell suspensions were stained with CD25-Alexa Fluor 488 and PE-Cy7 (PC61.5), CD4-PE and PerCp-Cy5.5 (RM4-5), CD8-allophycocyanin-Cy7 (53-6.7), Thy1.2-allophycocyanin-Cy7 (53-2.1), anti-MBP TCR clonotype biotin (3H12) (51), TCR Vβ11 (RR3-15), TCR Vα3.2 FITC (RR3-16), CD45.1 A700 and biotin (A20; all from eBioscience), and CD3-PerCp-Cy5.5 (145-2C11; BioLegend). Apoptotic cells were identified with 7-amino-actinomycin D and annexin V-allophycocyanin labeled in annexin V binding buffer (eBioscience). To prevent unspecific Ab capture by the Fc receptors, cells were incubated with anti-CD16/32 (clone 2.4G2) prior to staining. Samples were acquired on a FACSCanto or LSRII and analyzed with FlowJo.

CFSE labeling

Cells were resuspended at 1×10^6 cells/ml incubated at 37°C for 10 min in the presence of 5 μM CFSE. To stop the staining, cold complete medium was added.

Intracellular stainings

For intracellular cytokine staining, cells were isolated as described and stimulated for 4 h in complete culture medium containing 50 ng/ml PMA, 500 ng/ml ionomycin, and 10 μg/ml brefeldin A (Sigma-Aldrich). After staining of surface markers, cells were treated with a fixation and permeabilization kit (eBioscience) and stained with IFN-γ (XMG1.2), IL-17A (ebio 17B7), and Foxp3 (FJK165) Abs from BD Biosciences and eBioscience according to the manufacturers' recommendations.

In vitro Th17 polarization

MBP-specific $CD4^+$ T cells were sorted by magnetic separation with CD4 (L3T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from healthy 30-d-old TR^- mice. Cell purities were between 92 and 96%. The T cells were cultured for 5 d with bone marrow-derived dendritic cells (52) and 10 μg/ml MBP peptide (MedProbe, Oslo, Norway) in IMDM/5% FBS with 1 ng/ml TGF-β (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 10 ng/ml IL-1β (eBioscience), and 10 μg/ml anti–INF-γ (R46A2) with or without 10 μg/ml anti-CD4 (YTS177). At the end of the culture, cells were harvested and processed for flow cytometry.

Cytokine production assays

Splenocytes (1×10^6) were plated in 96-well flat-bottom plates and cultured in complete medium (RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPES, 1% penicillin/streptomycin, 1% sodium pyruvate, 0.1% 2-ME; Invitrogen) with 20 μg/ml peptide (MBP or $MOG_{35–55}$). Supernatants were recovered after 72 h and cytokines were quantified by sandwich ELISA for IL-10, IFN-γ (PrepoTech, London, U.K.), or IL-17 (R&D Systems).

Adoptive transfer of naive T cells

$CD4^+CD62L^+CD25^-$ T cells were purified from CD45.1 congenic 2D2 mice in two steps using microbeads (MACS; Miltenyi Biotec): first by negative selection using mAbs to CD8 (YTS169), B220 (RA3-6B2), Mac1 (CI:A3-1), and CD25 (7D4); followed by positive selection with anti-CD62L–coated beads, providing 80% $CD4^+$ T cells, of which 95% were $CD62L^+CD44^{lo}$ expressing the transgenic 2D2 TCR. Cells (1×10^5) were adoptively transferred into syngeneic B6 mice the day before immunization.

Viral plaque assay

Mice were infected with murid herpesvirus (MuHV)-4 by intranasal inoculation of 10^4 PFU in 20 μl PBS under halothane anesthesia. At days 7 (peak of lytic infection) and 14 (resolution of lytic infection), lungs were recovered and frozen at −80°C. On the day of the assay, lungs were homogenized in glutamine-free MEM complete media and submitted to 10× serial dilutions. To each dilution 5×10^5 BHK-21 cells were added. Viruses were left to adsorb for 1 h in six-well plates, at 37°C, followed by 4 d incubation after addition of 3 ml media. At day 4 of culture, the cell monolayer was fixed with 4% formaldehyde and stained with 0.1% toluidine blue. Viral plaques were counted with a Stemi SV6 magnifying glass (Zeiss). Virus titers were determined from the average of the number of counted viral plaques in duplicates.

Histology

Animals were perfused postmortem with 2% paraformaldehyde in PBS. Tissues were embedded in paraffin, and tissue sections were stained with H&E.

Statistical analysis

Statistical significance was determined using the two-tailed nonparametric Mann–Whitney U test, and p values of <0.05 were deemed significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Nondepleting anti-CD4 mAb prevents EAE

Previous studies primarily focused on the clinical amelioration afforded by CD4 mAb treatment on EAE in Biozzi AB/H (45), PL/J mice (44), and Lewis rats (32). Little is known regarding the underlying mechanisms. To study the impact of nondepleting CD4 mAb on the encephalitogenic T cell response, we chose the most widely used mouse model of EAE that is induced in C57BL/6 mice after immunization with MOG_{35-55}. Control mAb-treated mice (Fig. 1A) developed EAE on average 12 d after immunization causing hind limb paralysis to most animals (maximum disease score, 3 ± 0.4; Supplemental Table I). This paralytic disease was fully prevented when mice were treated with CD4 mAb on days 3 and 2 prior to immunization (Fig. 1, Supplemental Table I). Histological analysis indicated that disease prevention correlated with a reduced accumulation of immune cells in the CNS (Fig. 1B), including $CD4^+$ T cells (Fig. 1C). In vitro Ag recall experiments indicate that treatment with CD4 mAb reduces the intensity of the MOG_{35-55}-specific T cell response. Fourteen days after immunization, splenocytes from control mAb-treated mice produced significantly higher concentrations of IL-17 and IFN-γ in response to MOG restimulation in vitro as compared with immunized mice pretreated with CD4 mAb (Fig. 1D).

To test whether the efficacy of the treatment would persist after disease onset we treated mice with a single injection of 1 mg anti-CD4 mAb on the day of disease onset. As shown in Fig. 1E, the isotype control mAb-treated mice progressed from an initial tail atony to full paralysis of the hind limbs. Anti-CD4 mAb treatment halted disease progression, providing full disease remission 1 wk after treatment (Fig. 1E, Supplemental Table I).

Mice treated with nondepleting anti-CD4 mAb remain immunocompetent

To evaluate whether CD4 mAb-treated mice remain immunocompetent after treatment withdrawal, we evaluated the capacity of successfully treated mice to mount an antiviral immune response against a γ-herpesvirus infection. MuHV-4 causes transient pneumonia in B6 mice. This acute viral infection is efficiently cleared from the lungs after ~14 d by a cell-mediated immune response comprising both $CD8^+$ and $CD4^+$ T cells (53). We validated the implication of the adaptive immune response and the $CD4^+$ T cell response by infecting $RAG^{-/-}$ and MHC class $II^{-/-}$ mice, respectively (Fig. 2). The $RAG^{-/-}$ mice succumbed to uninhibited viral infection, causing increased viral titers 7 d postinoculation that proved lethal to all mice before day 14 (Fig. 2A). The role of $CD4^+$ T cells was revealed in MHC class $II^{-/-}$ mice where the absence of $CD4^+$ T cells increased the dissemination of MuHV as

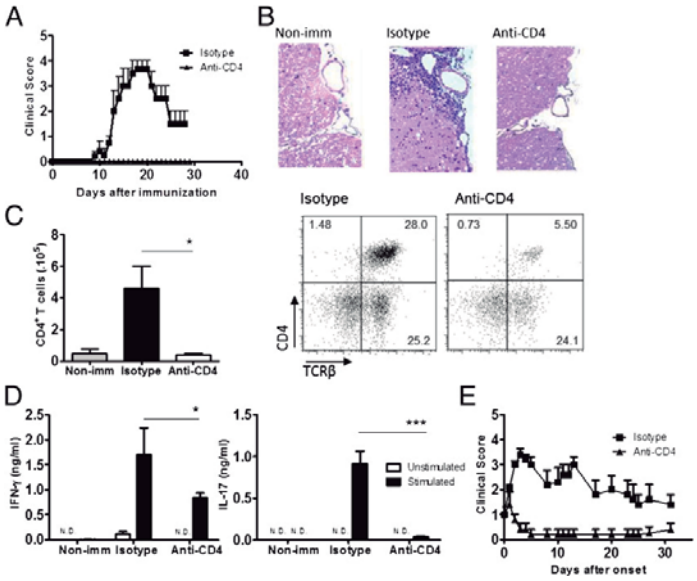

FIGURE 1. CD4 blockade treats active EAE in C57BL/6 mice. (**A**) CD4 mAb treatment prevents active EAE. Average clinical score of MOG_{35-55}-immunized mice treated with 1 mg nondepleting anti-CD4 mAb ($n = 5$; ▲) or isotype control mAb ($n = 5$; ■) at 3 and 2 d prior to immunization. The mean clinical score ± SEM is presented for one representative experiment out of three. (**B**) Parenchymal infiltration of inflammatory cells is prevented following CD4 blockade. H&E staining of transversal sections of the spinal cord 20 d after MOG_{35-55} immunization (original magnification ×400). Control mAb ($n = 3$; *middle column*) and nondepleting CD4 mAb ($n = 3$; *right column*) were injected 3 and 2 d prior to immunization. Aged-matched nonimmunized mice ($n = 3$; *left column*) were used as control. (**C**) CD4 mAb treatment prevents immune invasion of the CNS. Fifteen days after MOG_{35-55} immunization, CNS mononuclear cells were obtained by Percoll gradient from mice treated with an isotype control (black bar; $n = 5$) and anti-CD4 mAb (white bar; $n = 5$) 3 and 2 d before immunization. Age-matched nonimmunized mice (gray bar; $n = 5$) were used as control. $CD4^+TCR\beta^+CD8^-$ T cells were quantified by flow cytometry. The data represent the mean number of $CD4^+$ T cells per CNS ± SEM and two representative dot plots. (**D**) Anti-CD4 mAb treatment reduces the magnitude of the MOG_{35-55}-specific T cell response. Ag recall experiments were performed on splenocyte cultures 14 d after MOG_{35-55} immunization. The concentration of IFN-γ and IL-17 was measured 72 h after in vitro stimulation with 20 μg/ml MOG_{35-55} (filled bars) or medium alone (open bar). Mice were treated with control mAb ($n = 5$; *middle*) and anti-CD4 mAb ($n = 5$; *right*) 3 and 2 d prior to immunization. Age-matched nonimmunized mice were used as controls. Data represent mean cytokine concentration ± SEM. ND, not detected. (**E**) CD4 mAb therapy ameliorates established EAE. Average clinical score of MOG_{35-55}-immunized mice treated with a single injection of 1 mg nondepleting anti-CD4 mAb ($n = 5$; ▲) or isotype control mAb ($n = 5$; ■) on the day of disease onset. The data present the mean clinical score ± SEM after disease onset (day 0) and correspond to one representative experiment out of two. $*p < 0.05$, $***p < 0.001$.

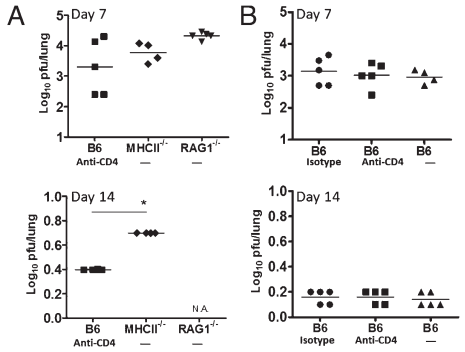

FIGURE 2. Mice treated with nondepleting anti-CD4 mAb remain immunocompetent. (**A**) To confirm the role of $CD4^+$ T cells in viral clearance, CD4-deficient MHC class $II^{-/-}$ and $Rag1^{-/-}$ mice were intranasally infected with 10^4 PFU MuHV-4. The viral load was quantified from lungs collected at days 7 and 14 that correspond to the peak of pneumonia and immune-mediated viral clearance. $Rag1^{-/-}$ mice died before day 14. MHC class $II^{-/-}$ mice did not resolve infection with the same efficacy as did mice treated with anti-CD4 30 d prior to infection ($n = 5$; $*p < 0.05$; NA, not applicable). (**B**) C57BL/6 mice were immunized with $MOG_{35–55}$ to induce EAE and treated with anti-CD4 mAb ($n = 5$; *middle*) or control mAb ($n = 5$; *left*) 3 and 2 d prior to immunization. Age-matched nonimmunized mice ($n = 5$; *right*) were used as control. Thirty days later the mice were infected as described above. The data show that anti-CD4–treated mice resolve infection with similar efficacy to control groups. Data are representative of two independent experiments.

indicated by higher viral titers 7 and 14 d postinfection (Fig. 2A). This model of viral clearance was used to test the immunocompetence of mice previously treated with anti-CD4 or control mAb 2 and 3 d prior to $MOG_{35–55}$ immunization. Thirty days after immunization mice were intranasally infected with MuHV-4 and the lungs were analyzed for virus content 7 and 14 d postinfection. Control B6 mice were infected with MuHV-4 in the absence of prior MOG-CFA immunization or CD4 blockade. As shown in Fig. 2B, mice that were initially treated with CD4 mAb were able to resolve the MuHV-4 virus infection detected at day 7 (Fig. 2B, *top*) by day 14 (*bottom*) with an efficiency equivalent to nonmanipulated control mice.

To further illustrate that nondepleting CD4 mAb treatment tolerizes only to Ags present at the time of treatment, we differentially applied CD4 mAb treatment to mice immunized with OVA and alum (Supplemental Fig. 1). We found that treatment with nondepleting anti-CD4 mAb prior to OVA immunization did not hamper subsequent immune responses to OVA (Supplemental Fig. 1A). In contrast, administration of OVA at the time of anti-CD4 treatment led to the induction of tolerance to this foreign Ag, which manifested as impaired production of OVA-specific IgG1 following subsequent OVA recall (Supplemental Fig. 1B).

CD4 blockade inhibits proliferation and functional polarization of MOG-specific T cells

To study the fate of encephalitogenic T cells at the single-cell level, we transferred a traceable population of naive $MOG_{35–55}$-specific $CD4^+$ T cells into CD45.2 B6 mice prior to EAE induction. These $CD62L^+CD25^-CD4^+$ T cells were purified from CD45.1 congenic, 2D2 transgenic mice expressing an $I\text{-}A^b$–restricted TCR ($V\alpha3.2$–$V\beta11$) specific for $MOG_{35–55}$ (50). As previously published, the transferred MOG-specific T cells differentiate into pathogenic Th1 and Th17 cells and expand 3700-fold by day 9 after immunization with $MOG_{35–55}$ (54). As shown in Fig. 3A, the recipient mice develop severe EAE that renders mice quadriplegic (maximum disease score, 4.3 ± 0.33) when injected with isotype control mAb at days 0, 2, and 4 after immunization with $MOG_{35–55}$. A similar treatment with nondepleting CD4 mAb fully protected the recipient mice from active EAE, as none of the animals developed any clinical signs of EAE (Fig. 3A). This is not because of depletion of the CD4 T cells, as the size of the CD4 T cell compartment was unaltered between isotype control- and nondepleting CD4 mAb-treated mice (Fig. 3B). However, when selectively enumerating the $MOG_{35–55}$-specific T cells a statistically significant reduction in the accumulation of 2D2 T cells was observed 9 d after immunization in mice treated with the CD4 mAb (Fig. 3C). This reduction was not associated with increased apoptosis, as the percentage of 2D2 T cells binding annexin V was similar in the isotype control- and CD4 mAb-treated groups 3 d after immunization with $MOG_{35–55}$ (Fig. 3D).

CD4 blockade inhibited T cell priming of the encephalitogenic T cell response as measured by CFSE dilution of the congenic MOG-specific 2D2 T cells. In the isotype control-treated mice most 2D2 T cells had undergone at least five cell divisions (51%) by day 3. In the CD4 mAb-treated mice this represented only 8% of the 2D2 T cells (Fig. 3E, 3F, Supplemental Fig. 2). The origin of this difference is likely to be bimodal, with less naive 2D2 T cells being activated after CD4 blockade, and second, that 2D2 T cells activated in the presence of CD4 blockade abort their expansion, as suggested by the limited number of cell divisions (Fig. 3E). Abortive T cell activation is characterized by T cell proliferation in the absence of effector differentiation. Consequently, we assessed whether CD4 blockade would prevent the commitment of naive 2D2 T cells to the Th1 and Th17 effector subsets 9 d after immunization. In vitro $MOG_{35–55}$ recall demonstrated a sizeable release of IL-17 and IFN-γ in lymph node cultures from isotype control-treated mice (Fig. 3G, Supplemental Fig. 2C, 2D). In CD4 mAb-treated mice the Ag recall response was downregulated, as indicated by the significant reduction in the release of effector cytokines (Fig. 3G). This was associated with a similar reduced frequency of 2D2 T cells expressing IFN-γ or IL-17 (Fig. 3H, Supplemental Fig. 2C, 2D), indicating that CD4 mAb treatment blocks the differentiation of naive 2D2 T cells into Th1 or Th17 effector cells. All data presented (Fig. 3B–H) were obtained from the draining inguinal lymph nodes.

Lastly, we addressed the contribution of regulatory $CD4^+$ T cells to the immunomodulatory impact of nondepleting CD4 mAb treatment. We assessed whether CD4 blockade during active immunization could favor the differentiation of naive T cells into induced $Foxp3^+$ Tregs. On day 9 after immunization with $MOG_{35–55}$ in CFA, we found an increased frequency of $Foxp3^+$ 2D2 T cells ($p < 0.05$), whereas the frequency of $Foxp3^+$ Tregs within polyclonal T cells remained unchanged (Fig. 3I).

Effector T cells are committed to apoptosis under anti-CD4 treatment

To study the mechanism by which nondepleting anti-CD4 mAbs treat established disease, as shown in Fig. 1E, we investigated the in vivo impact of anti-CD4 on terminally differentiated effector T cells. To this end, we passively induced EAE in C57BL/6 mice by adoptively transferring 14×10^6 MOG-specific 2D2 cells that were primed in vitro under Th1 polarization conditions (Fig. 4A) (55). Treating the recipient mice on days 0, 2, and 4 after Th1 transfer with anti-CD4 mAbs prevented the onset of disease in all treated mice (Fig. 4A). To study the fate of the transferred congenic 2D2 cells, we assessed their accumulation in the CNS and cervical lymph nodes 9 d after transfer, just prior to death of control mice (mortality on days 11–13). In the diseased isotype control-treated

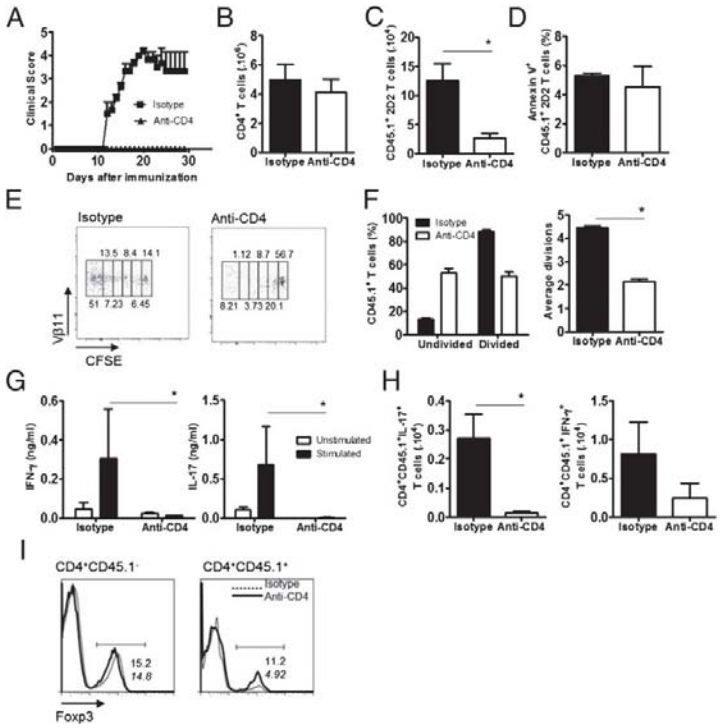

FIGURE 3. Anti-CD4 inhibits proliferation and differentiation of MOG-specific T cells. (**A**) Anti-CD4 mAb treatment prevents active EAE in model with traceable encephalitogenic T cells. Congenic 2D2 cells (1×10^5) were adoptively transferred into B6 mice and active EAE was induced by $MOG_{35–55}$ immunization the following day. Mice were treated with anti-CD4 ($n = 4$) and control mAb ($n = 4$) on days 0, 2, and 4. The mean clinical score ± SEM is presented for one representative experiment out of 3. (**B–F**) Anti-CD4 mAb treatment inhibits T cell priming. CFSE-labeled naive 2D2 T cells (1×10^6) were adoptively transferred into B6 mice prior to $MOG_{35–55}$ immunization. Flow cytometry analysis was performed on day 3 in the draining lymph nodes of mice treated with anti-CD4 mAb ($n = 4$; open bars) and control mAb ($n = 4$; filled bars) on days 0 and 2. By flow cytometry it is possible to distinguish the fate of encephalitogenic 2D2 cells ($CD45.1^+$) from polyclonal $CD4^+$ T cells. (B) Number of the total $CD4^+$ T cell compartment among draining lymph node cells. (C) Number of $CD45.1^+$TCR $V\alpha3.2^+CD4^+$ congenic 2D2 T cells in the draining lymph nodes. (D) Proportion of cells binding annexin V among CD45.1 TCR $V\alpha3.2^+CD4^+$ 2D2 T cells. (E) Representative dot plots showing CFSE dilution of $CD45.1^+$TCR $V\alpha3.2^+CD4^+$ 2D2 T cells after control mAb (*left panel*) or anti-CD4 mAb treatment (*right panel*). (F) CFSE data expressed as the frequency of 2D2 cells that did not divide or cells that had undergone cell divisions as assessed by CFSE dilution (*left plot*), and mean number of divisions of 2D2 T cells from mice treated with anti-CD4 (open bar) or a control mAb (filled bar). (**G–I**) Anti-CD4 mAb treatment inhibits effector T cell differentiation and favors $Foxp3^+$ Treg expansion. Naive 2D2 T cells (1×10^5) were transferred into B6 mice and active EAE was induced by $MOG_{35–55}$ immunization the following day. Mice were treated with anti-CD4 ($n = 4$) and control mAb ($n = 4$) on days 0, 2, and 4. Mice were analyzed 9 d after immunization. (G) Splenocyte cultures were restimulated with 100 μg/ml $MOG_{35–55}$ (filled bars) or cultured in the absence of Ag (open bars), and IL-17 release (*left*) and IFN-γ (*right*) were measured after 72 h by sandwich ELISA. (H) Ex vivo analysis of $CD4^+CD45.1^+$ 2D2 T cells for intracellular IL-17 (*left*) and IFN-γ (*right*) expressed as absolute number of cytokine-positive 2D2 T cells. (I) Histograms representing the frequency of $Foxp3^+$ cells within the polyclonal $CD4^+CD45.1^-$ T cells (*left*) and within MOG-specific 2D2 T cells ($CD4^+CD45.1^+$, *right*). The histograms represent cells from mice treated with anti-CD4 (dotted line) or an isotype control (thick line, frequency values in italics). The data represent the mean ± SEM of two independent experiments. $*p < 0.05$.

mice an average of 4×10^5 pathogenic 2D2 T cells could be recovered per CNS, whereas in the CD4 mAb-treated group the number of CNS infiltrating 2D2 T cells was significantly reduced (10-fold) (Fig. 4B). The transferred 2D2 T cells were also detectable in the cervical lymph nodes, but the cell numbers did not significantly differ from the control-treated group (Fig. 4C). However, in the CD4 mAb-treated group the fate of the pathogenic 2D2 T cells was significantly altered. As shown in Fig. 4D and 4E, up to 40% of transferred encephalitogenic T cells were undergoing apoptosis as measured by annexin V binding within congenic 2D2 T cells. Taken together, our data suggest that CD4 blockade triggers different mechanisms in naive and fully polarized Ag-specific cells. On naive T cells anti-CD4 mAb treatment prevents T cell priming, whereas on terminally differentiated T cells CD4 blockade predestines pathogenic T cells for apoptosis.

Anti-CD4 mAb treatment prevents fatal EAE in TCR-transgenic mice

MBP-specific TCR-transgenic mice that are Rag1-deficient (TR^-) spontaneously develop EAE between days 40 and 60 of life (49). All of the TR^- mice develop a disabling disease that progresses rapidly, leading to death within 5 d upon the initial clinical manifestations. This model permitted us to assess the efficiency of nondepleting CD4 mAb treatment during spontaneous neuroinflammation that develops without the need of adjuvant immunization. Treating the TR^- mice with two shots of 1 mg anti-CD4 mAb between days 30 and 35 of age protected all mice from EAE (incidence 0%; Supplemental Table I) when compared with isotype control-treated TR^- mice, all of which developed fatal EAE (incidence 100%; Fig. 5A). The efficacy of the non-depleting CD4 mAb treatment is such that even delaying treatment after

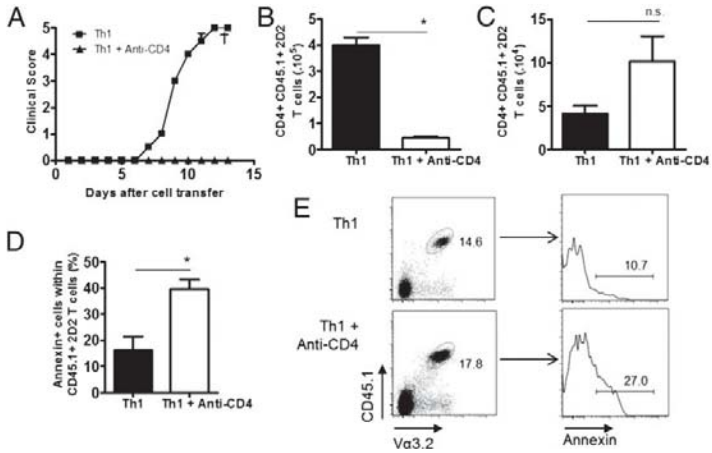

FIGURE 4. CD4 blockade prevents Th1-induced EAE through induction of apoptosis. (**A**–**D**) MOG-specific 2D2 T cells were polarized toward Th1 phenotype in vitro and transferred (14×10^6/mouse) into syngeneic B6 mice. (A) Average clinical score of mice treated with anti-CD4 mAb ($n = 4$; ■) or control mAb ($n = 4$; ▲) at the day of Th1 transfer and on days 2 and 4. The mean clinical score ± SEM is presented for one representative experiment out of two. (B–**E**) Nine days after 2D2 Th1 transfer anti-CD4 mAb ($n = 4$; open bars) or control mAb ($n = 4$; filled bars) treated mice were sacrificed and the number of $CD4^+CD45.1^+TCR\ V\alpha3.2^+$ T cells was determined by flow cytometry among CNS-infiltrating mononuclear cells (B) and in the cervical lymph nodes (C). (D) Proportion of cells binding annexin V among $CD45.1^+TCR\ V\alpha3.2^+CD4^+$ 2D2 T cells in the cervical lymph nodes. The data represent the mean ± SEM of two experiments. (E) Representative flow cytometry data from cervical lymph nodes of mice transferred with Th1-polarized cells in the absence of anti-CD4 (*top*) or subjected to CD4-blockade (*bottom*), showing higher frequency of cells binding annexin V in mice treated with anti-CD4 (histograms). $*p < 0.05$.

onset (clinical score 1) still permitted the progression of disease to be halted (Fig. 5B). In contrast, the isotype control treatment failed to delay the lethal disease progression, resulting in the death of all mice (Fig. 5B).

Similar to the active EAE setting, the overall $CD4^+$ T cell number is maintained following anti-CD4 treatment, confirming the nondepleting nature of the mAb (Fig. 5C). Unlike active EAE, we did not find evidence for increased apoptosis on autoantigen-specific T cells from anti-CD4–treated mice (Fig. 5D). Because TR^- mice are devoid of natural Tregs, we could conveniently evaluate the induction of Treg differentiation in anti-CD4–treated mice (Fig. 5E) (49). Two injections of 1 mg CD4 mAb in 30- to 35-d-old mice permitted the de novo generation of $CD4^+Foxp3^+$ T cells in cervical lymph nodes (Fig. 5E). The observed low percentage of induced $Foxp3^+$ Tregs is consistent with previous reports of Treg-mediated tolerance induced in TCR-transgenic Rag-deficient mice (52, 56).

To study the underlying mechanism, we evaluated whether in vitro stimulation of $CD4^+$ MBP-specific TR^- cells with MBP-loaded dendritic cells in the presence of anti-CD4 would alter the balance between the protective and proinflammatory Treg and Th17 populations. Addition of anti-CD4 to T cell cultures in Th17 polarizing conditions (in the presence of TGF-β and IL-6) led to a decrease in Th17 cells with a simultaneous increase of Treg frequency in a dose-dependent manner (Fig. 5F, 5G). This suggests that, in addition to recessive tolerance, nondepleting CD4 mAbs are able to convey dominant tolerance in animal models of CNS autoimmunity.

Discussion

The availability of different TCR-transgenic animal models of inflammatory demyelinating disease allows the study of mechanisms underlying putative tolerance-inducing regimens that influence different components of the immune system (57). In the last decade several studies have supported a role for T cell-directed therapies in the treatment of MS, where alemtuzumab is perhaps the most prominent example (58). Additionally, depleting (27, 28) and nonmitogenic (26, 59) anti-CD3 mAbs have been shown effective in inducing long-term protection from EAE. We used a nondepleting anti-CD4 mAb and found that CD4 blockade can lead to long-term protection from EAE while triggering different outcomes in naive or preactivated effector T cells.

We found that CD4 blockade was able to prevent the onset of EAE in MOG-CFA–immunized mice, as well as to impair disease progression when anti-CD4 was administered following the onset of clinical manifestations of the disease. Importantly, anti-CD4–treated mice remained fully competent to respond to unrelated Ags (Supplemental Fig. 1) and to mount protective immune responses toward a γ- herpesvirus (MuHV-4). However, at the time of anti-CD4 infusion there is a period where exposure to foreign Ags leads to tolerance rather than to protective immunity. Therefore, and depending on the pathogen characteristics, active infection when anti-CD4 is administered may interfere with effective pathogen clearance. In fact, in the early 1990s there were some promising studies with anti-CD4–induced EAE protection (32, 44–46) that led to some of the earliest clinical trials with therapeutic mAbs. However, the clinical development of this anti-CD4 therapy for MS was soon abandoned because of adverse effects related to CD4 T cell depletion (48). Our data show that, similar to what was previously shown in transplantation (60), autoimmune arthritis (61), and allergic diseases (62, 63), the therapeutic effect mediated by anti-CD4 mAb does not require direct T cell depletion.

To follow the fate of pathogenic T cells in vivo we adoptively transferred MOG-specific cells from 2D2 mice. It is well described that in EAE, the CNS suffers a massive infiltration by activated lymphocytes, namely CD4 effector T cells, which break the blood–brain barrier and recruit other proinflammatory cells to the site of inflammation, leading to an increased severity of the disease (64). We found that anti-CD4–treated mice did not show any significant infiltrates in the CNS while presenting a reduction of MOG-specific TCR-transgenic cells in the cervical lymph node

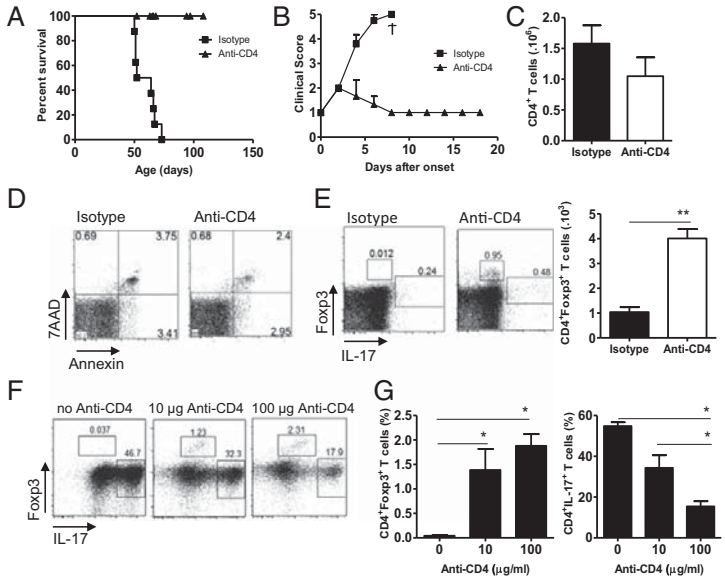

FIGURE 5. Anti-CD4 treatment prevents fatal EAE in TR$^-$ mice. (**A**) TR$^-$ mice develop fatal spontaneous EAE between days 40 and 60 of life. TR$^-$ mice were treated with 2 × 1 mg anti-CD4 between days 30 and 35 of life. Treated mice were protected from of the onset of EAE, whereas isotype-treated controls developed fatal disease ($n = 8$; $^{***}p < 0.001$). These data are representative of three independent experiments. (**B**) To investigate the impact of anti-CD4 treatment in established disease, TR$^-$ mice were treated with anti-CD4 or an isotype control upon the first manifestations of the disease. CD4-treated mice were protected from severe EAE whereas control animals died within days ($n = 5$; $^{***}p < 0.001$). (**C**) Number of CD4$^+$ T cells in cervical lymph nodes of anti-CD4-treated and control mice (NS). (**D**) Frequency of apoptotic cells, identified by binding of annexin V and 7-aminoactinomycin D (7AAD), in anti-CD4–treated and control mice (NS). (**E**) Number of CD4$^+$Foxp3$^+$ T cells in control and anti-CD4–treated mice, as well as representative dot plots. Mice treated with anti-CD4 showed significantly increased levels of Tregs ($n = 4$; $^{**}p < 0.01$). (**F** and **G**) In vitro cultures of TR$^-$ T cells stimulated with MBP, under Th17 polarization conditions. (F) Representative dot plots and (G) quantification of Foxp3$^+$ (*left*) and IL-17$^+$ (*right*) T cells. Addition of anti-CD4 mAb led to a decrease in Th17 commitment and an increase in the frequency of Foxp3$^+$ T cells expression in a dose-dependent way ($n = 4$; $^{*}p < 0.05$).

(the number of nontransgenic CD4 cells did not change, confirming the nondepleting nature of the mAb used). We also found that CD4 blockade acts by preventing T cell proliferation, as well as preventing the differentiation of effector cells producing proinflammatory cytokines. Our data show that the few encephalitogenic cells that proliferate in mice treated with anti-CD4 are committed to apoptosis, whereas in the control group they acquire IL-17– and IFN-γ–producing phenotypes (Supplemental Fig. 2). The molecular triggers that lead to apoptosis in the proliferating cells exposed to anti-CD4 mAb remain to be established. Additionally, we also found that among the transferred MOG-specific cells there is an expansion of Foxp3$^+$ T cells. These presumably induced Tregs can be especially important for the long-term maintenance of the tolerance state.

We then studied the effect of CD4 blockade on preactivated MOG-specific T cells, generally considered difficult to regulate. In fact, it was previously reported that MOG-specific Foxp3$^+$ Tregs are able to control naive neurotropic T cells but not activated effector T cells (65). We found that preactivated 2D2 cells do not cause pathology in the presence of CD4 blockade. Our data show that preactivated 2D2 cells under the cover of anti-CD4 fail to accumulate in the CNS, while being committed to apoptosis.

To further investigate the regulation of the balance between Tregs and effector T cells we used Rag1-deficient mice bearing a T cell repertoire exclusively composed of MBP-specific T cells and devoid of Foxp3$^+$ Tregs (TR$^-$) (49, 51). Despite the number of potentially aggressive cells these animals were protected from the development of EAE following a short course of nondepleting anti-CD4. We found protection from EAE correlated with the emergence of Foxp3$^+$ Tregs in the periphery. This is in agreement with several studies that support a role for Tregs in the control of CNS inflammation. In fact, some studies have shown that TR$^-$ mice can be protected from EAE following adoptive transfer of Tregs from anti-MBP TCR-transgenic Rag1$^{+/+}$ mice (51, 66, 67). Our in vitro studies have confirmed that blocking CD4 during the activation of TCR-transgenic T cells under Th17 polarizing conditions results in a decrease in IL-17–producing cells concomitantly with a greater frequency of Foxp3$^+$ cells in an anti-CD4 mAb dose-dependent way. It was indeed previously reported that strategies leading to a reduction of stimulatory signals received by the T cell (namely by reducing the concentration of agonist peptides or using CD4 blockade) can favor Foxp3 upregulation (52, 68). In all RAG$^{-/-}$ TCR-transgenic strains tested, we observed that the T cells that had acquired Foxp3 expression maintained normal levels of transgenic TCR expression.

Taken together, our data show that T cells under distinct stages of functional maturation respond differently to tolerance-inducing protocols. Therefore, it is likely that some reagents able to induce tolerance in naive T cells may be inadequate to effectively control preactivated T cells. Similarly, strategies that suppress terminally differentiated effector cells may be insufficient to induce long-term protection from the disease by promoting the peripheral induction of Tregs. In a similar way to what we have previously reported in an animal model of autoimmune arthritis (61), it appears that the key to long-term tolerance in EAE is the resetting of the abnormal effector Th1-Th17/Treg ratio. Importantly, naive and effector cells appear to be regulated differently: CD4 blockade prevents the proliferation and effector polarization of naive

T cells, while favoring Treg conversion; however, activated T cells are predominantly regulated by favoring their commitment to apoptosis.

Overall, our data show that effective tolerance-inducing strategies in autoimmunity will have to induce naive cells to acquire regulatory function important for the long-term maintenance of tolerance, while simultaneously disarming the preactivated effector T cells that may resist Treg-mediated regulation.

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Disclosures

The authors have no financial conflicts of interest.

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Regulation of the Germinal Center Reaction by Foxp3$^+$ Follicular Regulatory T Cells

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Follicular helper T (T_{FH}) cells participate in humoral responses providing selection signals to germinal center B cells. Recently, expression of CXCR5, PD-1, and the transcription factor Bcl-6 has allowed the identification of T_{FH} cells. We found that a proportion of follicular T cells, with phenotypic characteristics of T_{FH} cells and expressing Foxp3, are recruited during the course of a germinal center (GC) reaction. These Foxp3$^+$ cells derive from natural regulatory T cells. To establish the in vivo physiologic importance of Foxp3$^+$ follicular T cells, we used CXCR5-deficient Foxp3$^+$ cells, which do not have access to the follicular region. Adoptive cell transfers of CXCR5-deficient Foxp3$^+$ cells have shown that Foxp3$^+$ follicular T cells are important regulators of the GC reaction following immunization with a thymus-dependent Ag. Our in vivo data show that Foxp3$^+$ follicular T cells can limit the magnitude of the GC reaction and also the amount of secreted Ag-specific IgM, IgG1, IgG2b, and IgA. Therefore, Foxp3$^+$ follicular regulatory T cells appear to combine characteristics of T_{FH} and regulatory T cells for the control of humoral immune responses. ***The Journal of Immunology*****, 2011, 187: 4553–4560.**

Germinal centers (GCs) are temporary structures in secondary lymphoid organs in which Ag-specific B cells undergo extensive proliferation, class-switch recombination and somatic hypermutation (SHM), a unique process whereby they can acquire higher affinity for the Ag (1). Across several cell divisions, high-affinity GC cells are selected and differentiate eventually into either memory cells or long-lived plasma cells, providing the basis of a process called "affinity maturation of serum Abs". Long-term humoral immunity, as it is achieved in many prophylactic vaccines, is provided by those plasma cells and memory B cells and is a critical component to protect the body during subsequent infection (2).

In the last 15 y, an important body of information concerning the role of so-called follicular helper T (T_{FH}) cells in the GC reaction (GCR) has been obtained. This particular subpopulation of CD4$^+$ T cells is characterized by expressing high membrane levels of CXCR5 (the receptor of the follicular chemokine CXCL13, essential for follicular homing) and programmed death 1 [PD-1; a key molecule for B cell survival and selection, presumably through interaction with PD-ligand 2 (3)] and high levels of the transcription factor Bcl-6 (4, 5). Productive interactions between GC B cells and T_{FH} cells are mediated by cognate interactions through TCR and MHC class II–peptide interactions, CD40–CD40L, and ICOS–ICOS ligand (6). When CD40–CD40L interactions are prevented, the ongoing GCR stops and GCs dissolve within 24 h (6). Mice that are deficient in ICOS or ICOS ligand also have impaired GC formation and isotype switching (7). Triggering of the SHM process in GC B cells is also dependent on CD40–CD40L interactions (8). Immunized mice that lack T_{FH} cells show reduced numbers of germinal center B cells, as well as a reduction of Ag specific Ab (4). Besides direct B-T_{FH} contact, T_{FH} cells also contribute to the GCR through soluble mediators like IL-4 and IL-21 (9, 10). A recent report claimed distinct subpopulations of blood CXCR5$^+$ T cells can be found in humans producing cytokines characteristic of Th1, Th2, and Th17 responses (11).

Deregulation of proliferation, mutation, and differentiation in GCs can lead to detrimental outcomes, including oncogenesis and immunodeficiency (12, 13). Moreover, it has been shown that mutant B cells with self-specificity can be supported in GCs and contribute to autoimmunity (14). Therefore, regulation of the quality and quantity of plasma cells and memory B cell populations in GCs is important to prevent immunopathology.

How this regulation is achieved remains poorly understood. A potential mechanism for this could be the presence within the polyclonal follicular T cell population in GCs of some regulatory T cells with specificity for ubiquitous self-antigens. In agreement with that, theoretical modeling points to processes related to T cell proliferation as being the dominant steps in the GC dynamics (15). We thus propose the hypothesis that there are regulatory mechanisms affecting Ag-specific T_{FH} cells to prevent overly intense GCRs and/or production of autoantibodies. A possible basis for

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Abbreviations used in this article: GC, germinal center; GCR, germinal center reaction; LN, lymph node; NA, numerical aperture; PD-1, programmed death 1; PNA, peanut agglutinin; T_{FH}, follicular helper T; T_{FR}eg, follicular regulatory T cell; Treg, regulatory T cell.

some of the required GC regulation would be the involvement of Foxp3$^+$ regulatory T cells (Tregs) during the GCR.

Currently, little is known about Tregs in connection with GCs. Some Tregs are known to express high levels of CXCR5 and to display positive chemotaxis toward a CXCL13 gradient in vitro (16). Furthermore, CD25$^+$ T cells (presumably Tregs) were already shown to be present in the GCs of human tonsils and to suppress in vitro T cell activation and Ig production by B cells (16). In contrast, it was shown that within Peyer patches, Tregs can originate T_{FH} cells (17).

In this report, we show that in murine lymph nodes Tregs participate in the GCR, contributing to both the dynamics and the amplitude of the GCR. In addition, we show that those follicular Tregs have an effect on Ab production during a T-dependent immune response.

Materials and Methods

Mice and immunization

BALB/c, C57BL/6, CXCR5$^{-/-}$, OT2.Rag2$^{-/-}$ mice (TCR transgenic, OVA-specific), Foxp3^{gfp} knock-in mice (generously provided by A.Y. Rudensky, Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York, NY), and TCR$\alpha^{-/-}$ mice (B6;129S2-*Tcra*^{tm1Mom}/J, backcrossed to C57BL/6 background, from S. Tonegawa, RIKEN-MIT Center for Neural Circuit Genetics and RIKEN Brain Science Institute, Cambridge, MA) were maintained in specific pathogen-free facilities. Procedures were conducted in accordance with guidelines from the Animal User and Institutional Ethical Committees.

Unless otherwise stated in the text, animals were immunized i.p. with 20 μg OVA (Sigma, St. Louis, MO) previously run through a DetoxyGel column (Pierce, Rockford, IL) in 2 mg endotoxin-free aluminum hydroxide (alum; Alu-gel-S; Serva, Heidelberg, Germany).

Adoptive transfer

For adoptive cell transfers, single-cell suspensions from a pool of spleen and LNs were sorted based on expression of CD4, CXCR5, PD-1, CD25, and GFP (cells from Foxp3^{gfp} reporter mice) in a FACSAria (Becton Dickinson, Franklin Lakes, NJ), with doublet exclusion in all experiments. Unless stated otherwise in the text, 1×10^4 cells of the indicated cell population were injected intravenously into TCR-$\alpha^{-/-}$ mice. In all transfer experiments, mice were immunized with OVA-alum 1 d after adoptive transfer.

Flow cytometry

Single-cell suspensions of draining LNs were analyzed by flow cytometry using mAb targeting: PD-1 (J43), CD4 (L3T4), Thy1.2 (53-2.1), Foxp3 (FJK-16s), Bcl6 (GI191E), GITR (DTA-1), CD103 (2E7), CD69 (H1.2F3), CD25 (all from eBioscience), and anti-CXCR5 (2G8) and anti-Rat IgG2a (R35-95) (BD Bioscience, San Diego, CA). Foxp3 staining was performed using the Foxp3 Staining Set (eBioscience) following the manufacturer's instructions.

In vitro conversion

Sorted populations of 5×10^4 CD4 T cells were incubated for 3 d with 3 μg/ml plate-bound anti-CD3 (145-2C11; eBiosciences), 2 μg/ml soluble anti-CD28 (eBiosciences), and 5 ng/ml TGF-β (R&D Systems).

Suppression assay

Sorted populations of Foxp3^{gfp+} T cells were cocultured in Terasaki plates (Greiner, Frickenhausen, Germany) with γ-irradiated APCs, and Foxp3^{gfp-} effector cells (1:3:1 ratio). Cultures were supplemented with 2.5 μg/ml soluble anti-CD3 for 3 d, with the addition of 1 μCi ^{3}H-thymidine (Amersham, Sunnyvale, CA) in the last 12 h.

Confocal microscopy

Cryosections fixed in acetone (20 μm; Sigma) and vibratome sections from PFA-fixed tissue (50 μm; Sigma) were obtained from draining LNs. For Foxp3 staining sections were permeabilized with the Fix/Perm buffer from the Foxp3 staining Set (eBioscience). Samples were stained with the following primary Abs: rabbit anti-CD3, anti-Ki67, anti-GFP, anti-OVA; and rat anti-CD3 (Abcam), anti-IgM-TxRd (Southern Biotech, Birmingham, AL), anti-KJ-FITC (Caltag, Carlsbad, CA), anti–CD4-alexa647 (Serotech), peanut agglutinin (PNA)-FITC and PNA-bio (Vector, Burlingame, CA). We used the following as secondary Abs: anti–FITC-alexa488, anti-rabbit Ig-alexa488, anti-rabbit Ig-alexa647, anti-rat Ig-alexa633 (Invitrogen, Carlsbad, CA); and avidin-rhodamine (Vector) and streptavidin-DyLight488 from ThermoScientific (Rockford, IL). Images were acquired using an LSM710 confocal microscope (Zeiss, Jena, Germany) equipped with a ×5 (0.16 numerical aperture [NA]; Zeiss), ×10 (0.30 NA; Zeiss), ×20 (0.80 NA; Zeiss) and a ×40 (1.30 NA; Zeiss) objective. Image analysis was performed using the LSM image browser.

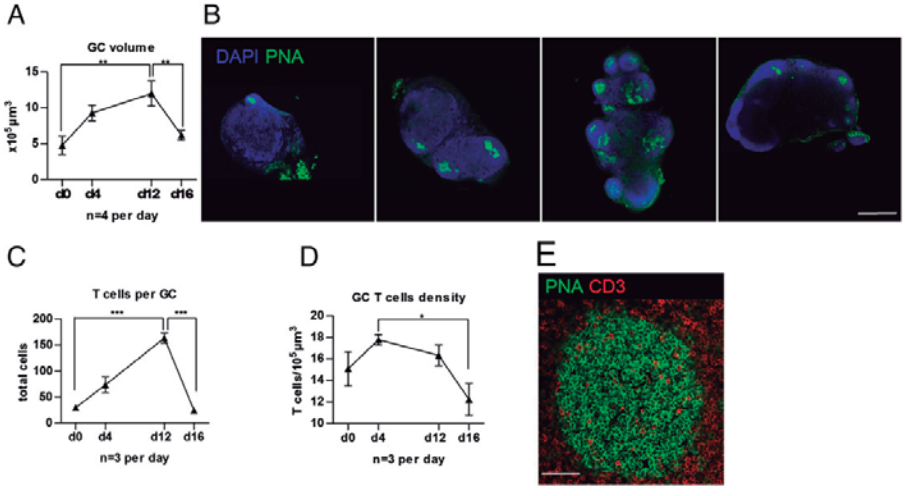

FIGURE 1. Kinetics of GCR and GC T cells. BALB/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed at the indicated time points, when cryosections of mesenteric lymph nodes were analyzed to follow the GCR. *A*, Average GC volume. GCs were identified by PNA staining and quantified by confocal microscopy. The volume (in cubic micrometers) was calculated from the Z-stack, multiplying the area by the depth of the GC. *B*, Representative stainings from nonimmunized mice, and mice sacrificed on days 4, 12, and 16 after immunization. Scale bar, 500 μm. *C*, Total number of T cells, and (*D*) density of GC T cells during a GCR as analyzed by confocal microscopy. GC T cells were identified as CD3$^+$ cells within the PNA area. *E*, Representative staining from an LN on day 12 after immunization. Data are representative of two independent experiments. Scale bar, 50 μm. ***$p < 0.001$ (Mann–Whitney nonparametric, two-tailed test).

Quantification of GC volume

The area of the ellipse circumscribing the GC was measured by the LSM image browser software. The volume was then calculated from the Z-stack, multiplying the area by the depth of the GC, and represented in cubic micrometers (i.e., a volume of 100,000 μm^3 is obtained in a structure of 100 × 100 μm and with a depth of 10 μm).

ELISA assay

Isotype-specific Ab titers in the serum were determined by ELISA on 96-well plates (BD Falcon) coated with 50 μg OVA (grade V; Sigma) in carbonate buffer (pH = 9.6) plus N_3Na 0.01%. Nonspecific binding was blocked with 1% BSA (AMRESCO, Solon, OH) in PBS plus Tween-20 0.05%. Sera were diluted 1:50 or 1:100 in PBST, and 3-fold serial dilutions of serum samples were incubated. Negative controls (normal mouse serum diluted 1:100) were included in all assays. The plates were washed, and 50 μl isotype-specific goat anti–mouse-HRP conjugates (Southern Biotech) was added. ELISAs were developed with ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (Sigma-Aldrich). The OD at 405 nm was determined after 4, 5, and 6 min with an automatic ELISA plate reader (Envision Multilabel Reader 2104, Perkin Elmer) and the highest values were recorded.

Statistical analysis

Statistical significance was determined using the two-tailed nonparametric Mann–Whitney *U* test, and $p < 0.05$ was deemed significant (in the figures, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Codevelopment of the GCR and GC T cells

To analyze GC T cells, BALB/c mice were immunized with OVA to trigger a GCR and sacrificed at days 0, 4, 12, and 16 to evaluate the initiation, peak, and decline of the GCR. Mesenteric LNs were

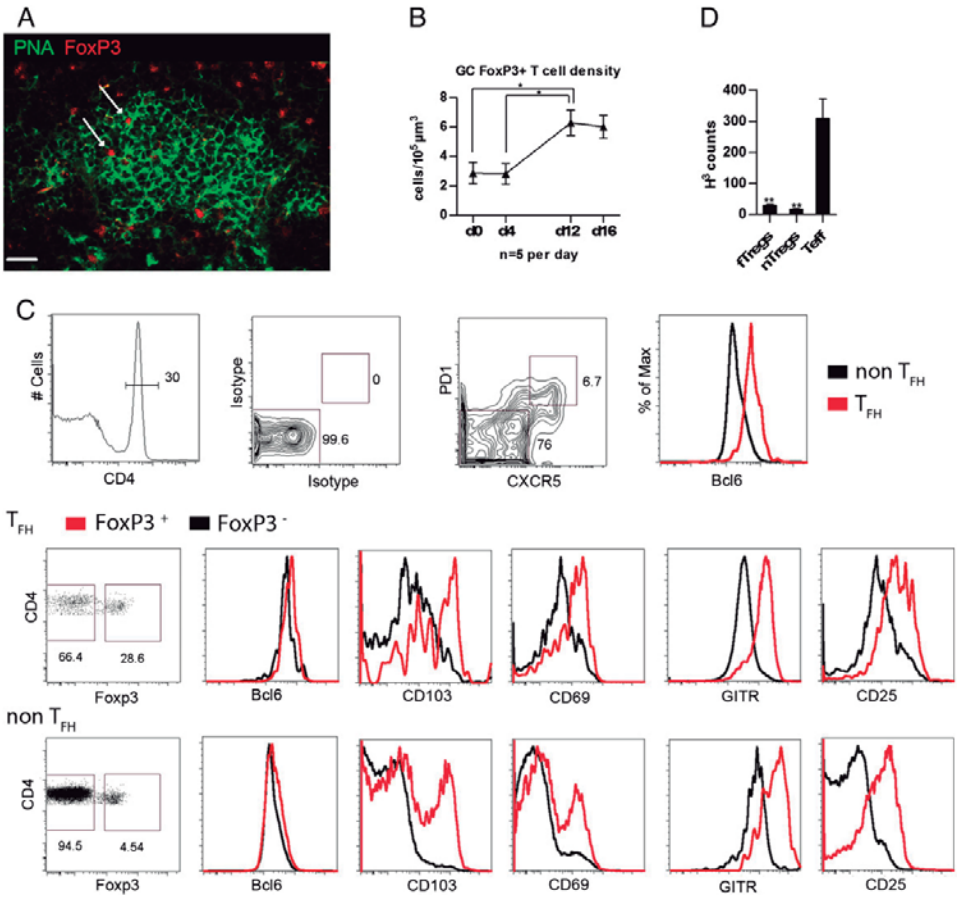

FIGURE 2. Foxp3$^+$ T cells can be found within the GC and represent a subpopulation of follicular T cells. BALB/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed at the indicated time points. *A*, Cryosections of LN were stained with PNA and anti-Foxp3 to identify Foxp3$^+$ cells within the GC. Scale bar, 20 μm. *B*, The density of Foxp3$^+$ T cells within the GC during the course of a GCR was quantified by confocal microscopy. The density corresponds to the total number of Foxp3$^+$ cells within the PNA$^+$ area divided by the measured GC volume. *C*, CD4$^+$ lymphocytes from LNs of mice immunized with OVA-alum 12 d before were studied by flow cytometry. T_{FH} cells were defined as being CXCR5$^+$PD1$^+$ as defined by the represented gate. The conventional extrafollicular T cells (non-T_{FH}) were defined as the CXCR5$^-$PD-1$^-$ cells. T_{FH} cells had higher expression of Bcl-6 (*top right*). When we studied the T cells within the T_{FH} gate (*middle row*) and non-T_{FH} gate (*bottom row*), we found that both populations had a proportion of cells expressing Foxp3. We compared the expression of the indicated molecules within the Foxp3$^+$ and Foxp3$^-$ populations of T_{FH} and non-T_{FH} cells. *D*, Follicular Foxp3$^+$ T cells (CD4$^+$CXCR5$^+$PD1$^+$Foxp3$^+$) and nonfollicular conventional Tregs (CD4$^+$CXCR5$^-$PD-1$^-$Foxp3$^+$) were sorted from the spleen and LNs of Foxp3^{gfp} reporter mice. Tregs (2×10^3) were cocultured in triplicate with γ-irradiated APCs and Foxp3^{gfp-} effector cells (1:3:1 ratio). Cultures were supplemented with 2.5 μg/ml soluble anti-CD3 Ab for 3 d, and ^{3}H-thymidine was added in the last 12 h. Data are representative of two independent experiments. $*p <$ 0.05, $**p < 0.01$ (Mann–Whitney nonparametric, two-tailed test).

collected, and 20-μm sections were stained with PNA and anti-CD3 to identify GCs and T cells. After immunization, the GCR peaked at ~day 12 or a few days before, as measured by the average GC volume using confocal microscopy (Fig. 1*A*, 1*B*).

The quantification of total T cells within the GCs revealed that the kinetics of T cell numbers parallels that of the global GCR (Fig. 1*C*). However, we calculated the density of T cells within the GC (i.e., number of T cells per unit of GC volume) because GC volume increases during GCR. We found that the T cell density was highest at day 4 after immunization (Fig. 1*D*), although the real peak is probably a few days later.

Follicular T cells contain a Foxp3$^+$ subset of suppressor cells

Given the observed changes in the density of T cells within the GC during a GCR, we investigated the putative presence of an active regulatory mechanism. Although Tregs were previously observed in germinal centers (16, 18), the precise nature of follicular Tregs, their relationship with extrafollicular counterparts, and their in vivo functional significance has remained unknown. We therefore investigated whether follicular Tregs represent a significant subpopulation of the T cells found in the GC.

As illustrated in Fig. 2*A*, Foxp3$^+$ cells were readily detectable in the GCs of mesenteric LNs. Interestingly, we found that the density of Foxp3$^+$ T cells peaked at day 12 after immunization (Fig. 2*B*), at the time when the density of T cells was reduced (Fig. 1*D*) and the contraction phase of the GCs was initiated. We confirmed these data using Foxp3^{gfp} knock-in mice (in which Foxp3 and GFP are fused together), showing a perfect overlap of Foxp3 staining and GFP expression (Supplemental Fig. 1*A*). By analyzing nonconsecutive sections from the same LN, we also established the reliability of the quantitative approach that we used (Supplemental Fig. 1*B*).

To further characterize the GC Foxp3$^+$ cells that we observed by confocal microscopy, we analyzed follicular T cells of mesenteric LNs by flow cytometry. We confirmed the existence of a Foxp3$^+$ subpopulation within follicular T cells (Fig. 2*C*). We found that follicular Foxp3$^+$ T cells share several markers with conventional Tregs, such as high levels of CD25, GITR, or CD103, but also retain the expression of molecules characteristic of T_{FH} cells, such as CXCR5 and PD-1, and the lineage-specific transcription factor Bcl-6 (Fig. 2*C*). Furthermore, we found that FACS-sorted follicular Foxp3$^+$ T cells (sorted based on CD4, PD-1, CXCR5, and Foxp3^{gfp} expression) had similar immune suppressive potential as nonfollicular Foxp3$^+$ T cells (Fig. 2*D*).

As a result, Foxp3$^+$ T cells found in GCs and B cell follicles appear to represent a subpopulation of follicular T cells, bearing a distinctive phenotype from the extrafollicular conventional Tregs. Importantly, Bcl-6 was recently identified as the master transcription factor for the T_{FH} lineage, by turning on a widespread gene repressor program acting on key transcriptional regulators of other Th cell lineages, namely Tbet (Th1) and RORγt (Th17), as well as a large number of miRNAs (4, 5, 19). It is important to note, however, that a repressive function toward the Treg lineage, or its key regulator Foxp3, was not found.

Increased density of Foxp3$^+$ GC T cells correlates with their increased proliferation as the GCR develops

We next assessed the proliferative behavior of GC Foxp3$^+$ T cells by quantifying the number of cells positive for the proliferation marker Ki67. As shown in Fig. 3, the frequency of proliferating (Ki67$^+$) Foxp3$^+$ cells within the GC is greater than the frequency of proliferating Foxp3$^+$ cells outside the GC. In addition, there is a significant increase of proliferation at day 12 after immunization—a time that, as discussed above, correlates with an increase

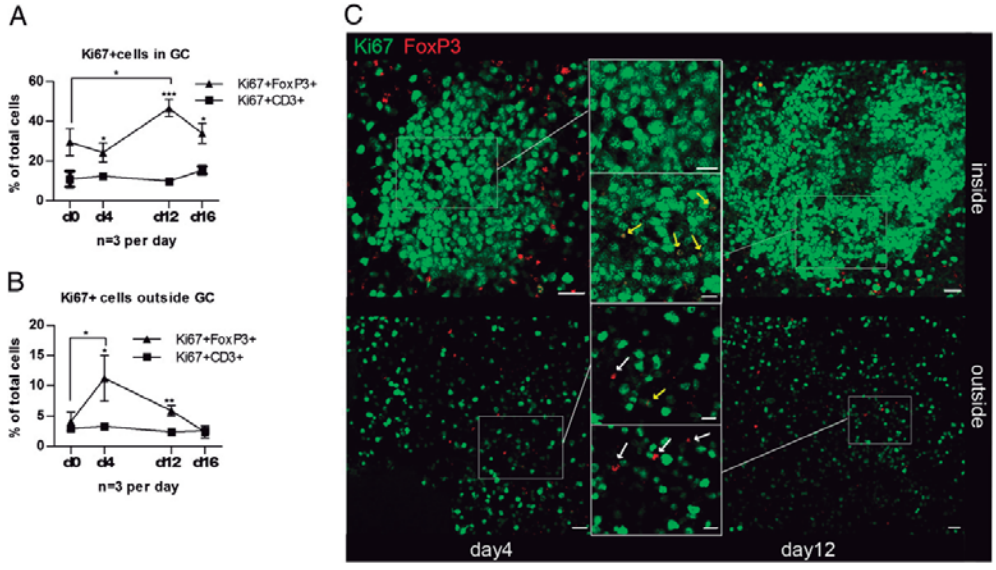

FIGURE 3. Kinetics of proliferation of T cells and Foxp3$^+$ T cells during the GCR. BALB/c mice were immunized with OVA-alum as described in the text. The frequency of proliferating T cells was quantified by confocal microscopy based on Ki67 expression. *A*, Proliferating GC Foxp3$^+$ cells and GC T cells were identified as Ki67$^+$Foxp3$^+$ cells and Ki67$^+$CD3$^+$ cells, respectively, within the PNA area and are depicted as a percentage of the total Foxp3$^+$ or total CD3$^+$ cells identified in the GC. *B*, A similar analysis was performed for T cells outside the GC. *C*, Representative stainings of the proliferation of GC T cells. Yellow arrows point to Ki67$^+$Foxp3$^+$ cells, white arrows to Ki67$^-$Foxp3$^+$ cells. Data are representative of two independent experiments. Scale bars, 20 μm (*insets*, 10μm). $*p < 0.05$, $**p < 0.01$ (Mann–Whitney nonparametric, two-tailed test).

in the frequency of Foxp3$^+$ cells within the GC. It is noteworthy that at day 4 after immunization, the proliferation of Foxp3$^+$ cells increases significantly outside the GC. On the contrary, the frequency of proliferating cells within total GC T cells does not change during the GCR.

Foxp3$^+$ GC T cells regulate the magnitude of GCR

After observing an increased frequency of Foxp3$^+$ GC T cells at day 12 after immunization, we assessed the functional significance of such cells for the regulation of GCR. For this purpose we used TCR-α$^{-/-}$ mice, which lack α/β T cells and Tregs, and reconstituted those mice with OVA-specific CD4 T cells from OT2.Rag2$^{-/-}$ mice (that also lack Foxp3$^+$ T cells). The OT2 cells were transferred alone or with sorted Tregs. We took advantage of Foxp3^{gfp} knock-in reporter mice to isolate a population of Foxp3^{gfp+} Tregs by flow cytometry. The recipient mice were immunized with OVA, and GCR was followed at different time points. We studied GC resolution at day 20, instead of day 16, to have better discrimination concerning a putative impact on the serum titer of switched Igs.

Because TCRα$^{-/-}$ mice are known to show an impaired ability to develop GCs owing to their lack of α/β T cells (20), we first wanted to ensure that TCRα$^{-/-}$ mice could develop GCs after adoptive cell transfer of Ag-specific cells. Lymph node sections from TCR-α$^{-/-}$ mice transferred with OT2 cells (CD4$^+$ T cells from OT2.Rag2$^{-/-}$ mice) and immunized with OVA-alum were analyzed for the presence of GCs by staining with PNA and anti-IgM. This study revealed the ability of OT2 cells to mount a GCR (Fig. 4*A*), with kinetics having a relatively high magnitude (Fig. 4*B*). However, cotransfer of Foxp3$^+$ Tregs together with OT2 cells, significantly reduced the magnitude of the GCR (Fig. 4*B*). Consistent with this finding, we also found that the adoptive transfer of Foxp3$^+$ Tregs had an effect on Ab production, leading to lower serum titers of secreted OVA-specific IgG1, IgM, IgG2b, and IgA (Fig. 4*C*). The Ab titres were reduced approximately 1 log by day 20, but with mice transferred with Tregs still showing lower serum titres (Fig. 4*D*).

However, given the known ability of Foxp3$^+$ Tregs to suppress T cells responses, it was possible that the observed regulation was acting on the generation of helper T cells during their early stage of activation taking place in the T zone of the LN, and not within the GC. To address this issue we took advantage of CXCR5$^{-/-}$ mice. These mice provided the tool to discriminate between the in vivo physiologic effects of Tregs within and outside the follicle, because Tregs from CXCR5$^{-/-}$ mice have a function similar to wild type Tregs but cannot enter the follicle. Therefore, we repeated the experiment performing an adoptive transfer of OT2 cells into TCR-α$^{-/-}$ mice. Flow cytometry-sorted CD4$^+$ CD25^{bright} T cells (that we confirmed were predominantly Foxp3$^+$) from Foxp3^{gfp} or CXCR5$^{-/-}$ mice were transferred with the OT2 cells. As anticipated, Tregs from CXCR5$^{-/-}$ mice could not be found within the GC, while the effector OT2 cells could still be found in the GC (Fig. 4*E*, 4*F*). Moreover, the ratio of effector to regulatory cells observed in the GC of TCR-α$^{-/-}$ mice transferred with T cells was similar to that in wild type conditions (Fig. 4*E*, 4*F*), although with a smaller number of cells. The ratio of follicular Tregs to T effector cells is not too different from the ratio observed within conventional extrafollicular populations (i.e., ~10%). Remarkably, we were able to show the physiologic role of follicular Foxp3$^+$ T cells, because we found that adoptively transferred CXCR5$^{-/-}$ Tregs could not reproduce the phenotype observed with wild type Tregs (Fig. 4*G*). Only mice transferred with Tregs able to express CXCR5 had detectable Tregs within the GC (Fig. 4*H*), whereas both groups of mice had transferred Tregs in locations outside the follicle.

To address whether follicular Foxp3$^+$ T cells derive from nonfollicular precursors, we repeated the adoptive cell transfer described above, but now using nonfollicular (PD-1$^-$CXCR5$^-$) Tregs sorted from wild type or CXCR5$^{-/-}$ mice. We found that at day 12 after immunization, mice transferred with Tregs from CXCR5$^{-/-}$ donors had greater GCs (Fig. 4*I*). Furthermore, mice transferred with Tregs deficient in CXCR5 had greater titres of serum Igs (Fig. 4*J*). It should be noted, however, that this defect in regulation of isotype switch and Ab production by CXCR5$^{-/-}$ Tregs was not complete, probably representing a degree of extrafollicular regulation and possibly affecting the availability of T_{FH} cells within the follicle.

Considering these results, it appears that CXCR5 expression by Treg cells is essential to endow them with the ability to control the GCR. As a result, we propose naming this Foxp3$^+$ T cell subset from GCs that express PD-1, CXCR5, and Bcl-6, as regulatory follicular T cells ($T_F$regs).

Follicular Foxp3$^+$ T cells derive from conventional Treg cells

We then investigated whether Foxp3$^+$ follicular T cells derive from conventional T cells or from natural Tregs that acquire a follicular phenotype. We confirmed, as anticipated, that the thymus does not contain a population of Foxp3$^+$ T cells with follicular characteristics (Fig. 5*A*), indicating that the follicular Foxp3$^+$ T cells acquire their phenotype in the periphery.

To establish the origin of follicular Foxp3$^+$ cells, we adoptively transferred sorted T_{FH} or conventional CD25$^-$CD4$^+$ T cells from wild type mice, together with PD-1$^-$CXCR5$^-$Foxp3^{gfp+} natural Tregs into TCRα$^{-/-}$ mice, that were subsequently immunized with OVA-alum. Because adoptive transfer of a CD4 population devoid of Tregs leads to inflammatory bowel disease (21), control mice that did not receive Tregs were transferred with OT2 cells, as these TCR-transgenic T cells do not lead to autoimmunity in the absence of Tregs. We found in both cases that the majority of follicular Foxp3$^+$ T cells recovered from the TCR-α$^{-/-}$ mice had derived from the transferred Treg population, where GFP was expressed together with Foxp3 (Fig. 5*B–D*). Intriguingly, that fraction was larger when T_{FH} cells were transferred together with Foxp3^{gfp+} Tregs, suggesting a role for T_{FH} cells in recruiting Foxp3$^+$ follicular T cells, perhaps by promoting their proliferation. This issue remains to be further investigated.

Furthermore, we sorted T_{FH} and conventional CD4 T cells that were stimulated in vitro with plate-bound anti-CD3 in the presence of anti-CD28 and TGF-β—conditions that are known to induce Foxp3 expression (22, 23). We found that T_{FH} cells are resistant to conversion toward a Foxp3$^+$ phenotype (Fig. 5*E*).

These data suggest that $T_F$regs derive predominantly from natural Tregs, whereas T_{FH} cells are not amenable to TGF-β–dependent conversion into Foxp3$^+$ regulatory cells.

Discussion

Owing to the absence of experimental data, there are various mathematical models attempting to explain the regulation and termination of the GCR. One hypothesis suggests an increasing impairment of the engagement of B cell receptors and Ag through either Ag consumption (24) or Ag masking (25). Both of these concepts assume that GC termination is regulated by the amount of Ag presented within the GC by follicular dendritic cells. Another hypothesis suggests GC termination is caused by increased differentiation of GC B cells toward memory and plasma cells because of signals from follicular dendritic cells (15). A third

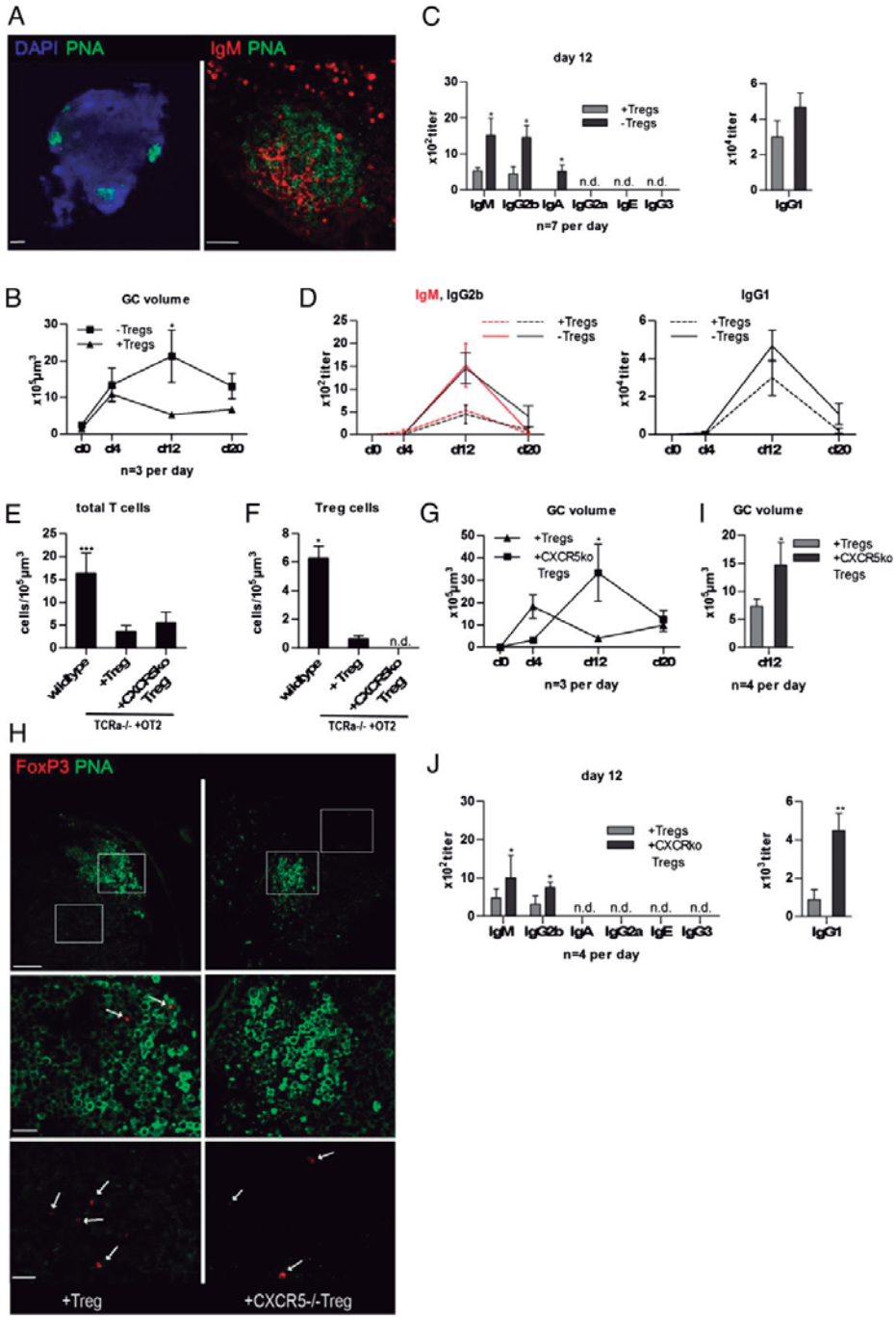

FIGURE 4. Follicular Foxp3$^+$ T cells regulate the magnitude of GCR in vivo. *A*, TCR$\alpha^{-/-}$ mice were adoptively transferred with 10^4 OVA-specific T cells from OT2.Rag2$^{-/-}$ mice. Some mice also received an equal number of Foxp3^{gfp+} T cells. All mice were immunized with OVA-alum. The micrographs show GCs in mice that did not receive Foxp3$^+$ T cells at day 12 after immunization. Scale bars, 200 μm (*left*), 50 μm (*right*). *B*, Quantification of the GC volume from LNs of mice immunized with OVA-alum in the presence or absence of adoptively transferred Foxp3$^+$ T cells. GCs were identified as a PNA$^+$ region within the follicle marked by IgM. *C* and *D*, Serum titres of OVA-specific Igs of different classes were determined by ELISA at different times after immunization. In the absence of Tregs, the mice produced significantly higher levels of IgM, IgG1, IgG2b, and IgA. *E*, Quantification of total CD4$^+$ T cells and (*F*) Foxp3$^+$ Treg cells within GCs showing that, following adoptive cell transfers of defined T cell populations into T cell-deficient mice, there was a reduced number of both T cells and Tregs compared with wild type mice, although their ratio was roughly maintained. *G*, TCR-$\alpha^{-/-}$ mice were adoptively transferred with OT2 T cells and Tregs sorted from wild type or CXCR5$^{-/-}$ mice and immunized with OVA-alum as (*Figure legend continues*)

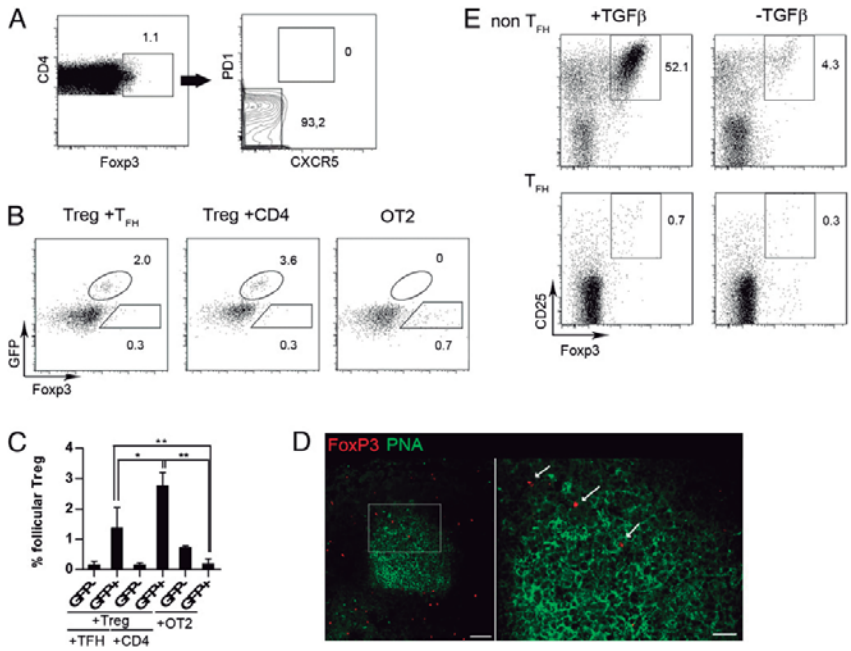

FIGURE 5. Follicular Foxp3$^+$ T cells originate from conventional Treg cells. *A*, Among CD4$^+$Foxp3$^+$ thymocytes, we could not find cells expressing CXCR5 and PD1. *B*, TCR-$\alpha^{-/-}$ mice were adoptively transferred with equal numbers of PD-1$^-$CXCR5$^-$Foxp3$^+$ Tregs sorted from Foxp3^{gfp} knock-in mice and CD4$^+$CD25$^-$ T cells (T_{FH}: PD-1$^+$CXCR5$^+$ or CD4: PD-1$^-$CXCR5$^-$) from B6 mice. Control mice were transferred with Foxp3$^-$ OT2 cells alone. All mice were immunized with OVA-alum. The dot plots represent follicular CD4$^+$ T cells, gated in the PD-1$^+$CXCR5$^+$ region, analyzed for Foxp3 and GFP expression. Representative data are from four mice per group. *C*, The majority of Foxp3$^+$ cells are also GFP$^+$, indicating that they are derived from adoptively transferred Tregs (from Foxp3^{gfp} mice). $n = 4$; $*p < 0.05$, $**p < 0.01$, (Mann–Whitney nonparametric, two-tailed test). *D*, Micrograph showing Foxp3$^+$ T cells in the GC of adoptively transferred mice (white arrows). Scale bar, 50 μm (*left*), 20 μm (*right*). *E*, Foxp3^{gfp-} T_{FH} (sorted as CD4$^+$PD-1$^+$CXCR5$^+$) and non-T_{FH} cells (sorted as CD4$^+$PD-1$^-$CXCR5$^-$) were cultured for 3 d with plate-bound anti-CD3 in the presence or absence of 5 ng/ml TGF-β. The frequency of cells converted into Foxp3$^+$ was analyzed at the end of the culture ($n = 3$, representative of two independent experiments).

hypothesis proposes that signaling from T cells is the responsible for GC termination (15). After observing an increase in $T_F$reg numbers in the GC toward the end of the GCR, we suggest a wider scenario whereby this cell population has a role in controlling the magnitude and duration of the GCR.

The lack of Tregs in our transfer system profoundly modified the outcome of the GCR, as those mice showed larger GCs at day 12, and greater production of different classes of Ig than in the presence of Tregs. Moreover, using Tregs isolated from CXCR5$^{-/-}$ mice, we created a system with Tregs that are unable to gain access to GC follicles. As a result, the data showed that it is specifically the $T_F$regs that control the GCR. Although our data do not prove unequivocally a role for $T_F$regs in the termination of the GCR, they clearly show that $T_F$regs affect the magnitude of this reaction from midterm on. To date, it is not clear whether this regulation operates by controlling effector T_{FH} cells (16), and by that indirectly affecting GC B cells, or if that regulation acts directly on GC B cells or on both T_{FH} and B cells (18).

A second outstanding question concerns the origin of $T_F$regs. We could not detect Foxp3$^+$ cells with a T_{FH}-like phenotype in the thymus. Furthermore, when we transferred OT2 cells into TCR$\alpha^{-/-}$ mice, we could not find in vivo conversion of those Foxp3$^-$ cells into $T_F$regs. When we transferred natural Tregs together with CD4 T cells, some of the natural Tregs (the only ones expressing Foxp3^{gfp} in those mice) acquired access to the follicles and phenotypic follicular characteristics. Therefore, we believe it is likely that, in the same way that natural Tregs can originate T_{FH} cells in the PP (17), some natural Tregs can also differentiate toward a $T_F$reg phenotype in LNs and the spleen. This suggestion has important implications for understanding how the dynamics of T and B cells within GCs is controlled.

GC deregulation or malfunctions are linked to the onset of severe diseases, as cancer and autoimmunity (26). Our data show that follicular T cells include a new regulatory subpopulation, which by controlling the GCR may ultimately have an effect on preventing such pathologies.

described in *A*. The GC volume was quantified as detailed in the text. Data are representative of two independent experiments. *H*, Representative micrographs showing that it was possible to identify Foxp3^{gfp+} T cells within day 12 GCs of mice adoptively transferred with Tregs, but not in mice transferred with CXCR5$^{-/-}$ Tregs, where Tregs remained outside the follicle. Scale bars, 100 μm (*top row*), 20 μm (*bottom two rows*). *I*, TCR-$\alpha^{-/-}$ mice were adoptively transferred with OT2 cells together with nonfollicular (CXCR5$^-$PD-1$^-$) Tregs sorted from wild type or CXCR5$^{-/-}$ mice and immunized with OVA-alum as described in *A*. The GC volume was quantified at day 12 as detailed in the text. *J*, Serum titers of OVA-specific Igs of different classes were determined by ELISA at day 12 after immunization. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Mann–Whitney nonparametric, two-tailed test). n.d., not detected.

Note added in proof. While our manuscript was being reviewed, two publications have reported the identification of follicular Foxp3$^+$ regulatory T cells and their functional significance in regulating GCR (27, 28). Those reports complement the findings described in this study.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure 1. *Validation of the quantitative studies.* (A) Cryosections from LNs of Foxp3^{gfp} knock-in mice, previously immunized with OVA-alum, were stained with anti-Foxp3 and anti-GFP antibody to confirm that both molecules allowed the identification of the same cells. Bar: 50 µm. (B) Balb/c mice were immunized with OVA-alum i.p. and non-consecutive cryosections from the same LN were studied at different time points. The quantified cells were identified as the Foxp3$^+$ cells within the PNA area.

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Prevention of House Dust Mite Induced Allergic Airways Disease in Mice through Immune Tolerance

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Abstract

Allergic airways disease is a consequence of a Th2 response to an allergen leading to a series of manifestations such as production of allergen-specific IgE, inflammatory infiltrates in the airways, and airway hyper-reactivity (AHR). Several strategies have been reported for tolerance induction to allergens leading to protection from allergic airways disease. We now show that CD4 blockade at the time of house dust mite sensitization induces antigen-specific tolerance in mice. Tolerance induction is robust enough to be effective in pre-sensitized animals, even in those where AHR was pre-established. Tolerant mice are protected from airways eosinophilia, Th2 lung infiltration, and AHR. Furthermore, anti-CD4 treated mice remain immune competent to mount immune responses, including Th2, to unrelated antigens. Our findings, therefore, describe a strategy for tolerance induction potentially applicable to other immunogenic proteins besides allergens.

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Introduction

The control of deleterious immune responses causing diseases, such as allergy, autoimmunity and transplant rejection, has been one of the main objectives of immunologists. Moreover, the global prevalence of this type of diseases has been steadily increasing.

Several strategies have been recently described to induce tolerance to allergens thus preventing allergic airways disease [1,2,3,4]. In brief, they can rely on the induction of dendritic cell (DC) populations or regulatory T cells (Treg) able to control pathologic T cell clones, in a process where IL-10 and TGF-β can participate [1,5,6,7,8,9,10]. In addition, disease prevention may be achieved by skewing the immune response from a Th2 to a Th1 phenotype [11].

In fact, the realization of the critical importance of T cells in the pathogenesis of allergic airways disease was well demonstrated by studies where anti-CD4 monoclonal antibodies (MAbs) causing the depletion of this T cell subset could prevent the disease in mice [12]. Such pre-clinical studies with CD4 T cell depletion provided the rationale for clinical trials with depleting anti-CD4 MAbs where the short-term benefit observed was probably associated with transient immune suppression [13]. As a consequence, the interest has shifted towards MAbs capable of blocking molecular interactions but without leading to direct cell lysis.

Some reports have shown prevention of allergic airways disease following the blockade of T cell co-stimulatory or co-receptor molecules with non-depleting MAbs, but it remains unclear whether long-term antigen-specific tolerance is achieved or what are the mechanisms involved [14,15,16,17]. We now describe CD4 blockade at the time of exposure with a model antigen, ovalbumin (OVA), or a clinically relevant allergen, house dust mite (HDM), can induce antigen-specific tolerance and protection from allergic airways disease. The mechanism leading to antigen-specific tolerance without affecting protective immune responses (including Th2-type responses) to additional antigens is independent of a switch between a Th2-type and Th1-type immune response. Since CD4 blockade is achieved with a non-depleting MAb, T cells not activated by the antigen remain unaffected to mount protective immune responses towards unrelated antigens at a later time.

Tolerance induction by CD4 blockade is robust enough to be effective in pre-sensitized animals and even in animals where AHR was previously established. The tolerant mice show protection from allergic manifestations elicited by intranasal exposure to the antigen: they do not develop airways eosinophilia, goblet cell hyperplasia, production of Th2 cytokines in the lung, production of antigen-specific IgE or IgG1, and, importantly, do not develop airway hyperreactivity (AHR) in response to inhaled methacholine (MCh).

Results

Co-receptor blockade with non-depleting anti-CD4 MAb prevents allergic sensitization in mice

Using a well established murine model of allergic airways disease we sought to determine if non-depleting MAbs targeting the T cell co-receptor molecule CD4 were effective in preventing allergic sensitization with HDM or a model antigen (OVA).

BALB/c mice were sensitized with two i.p. injections of OVA-alum or HDM-alum on days 1 and 14, and challenged with 50 μg OVA or HDM i.n. on days 20, 21 and 22 (Figure 1A). Experimental groups were treated with 1 mg i.p. of anti-CD4 or an isotype control

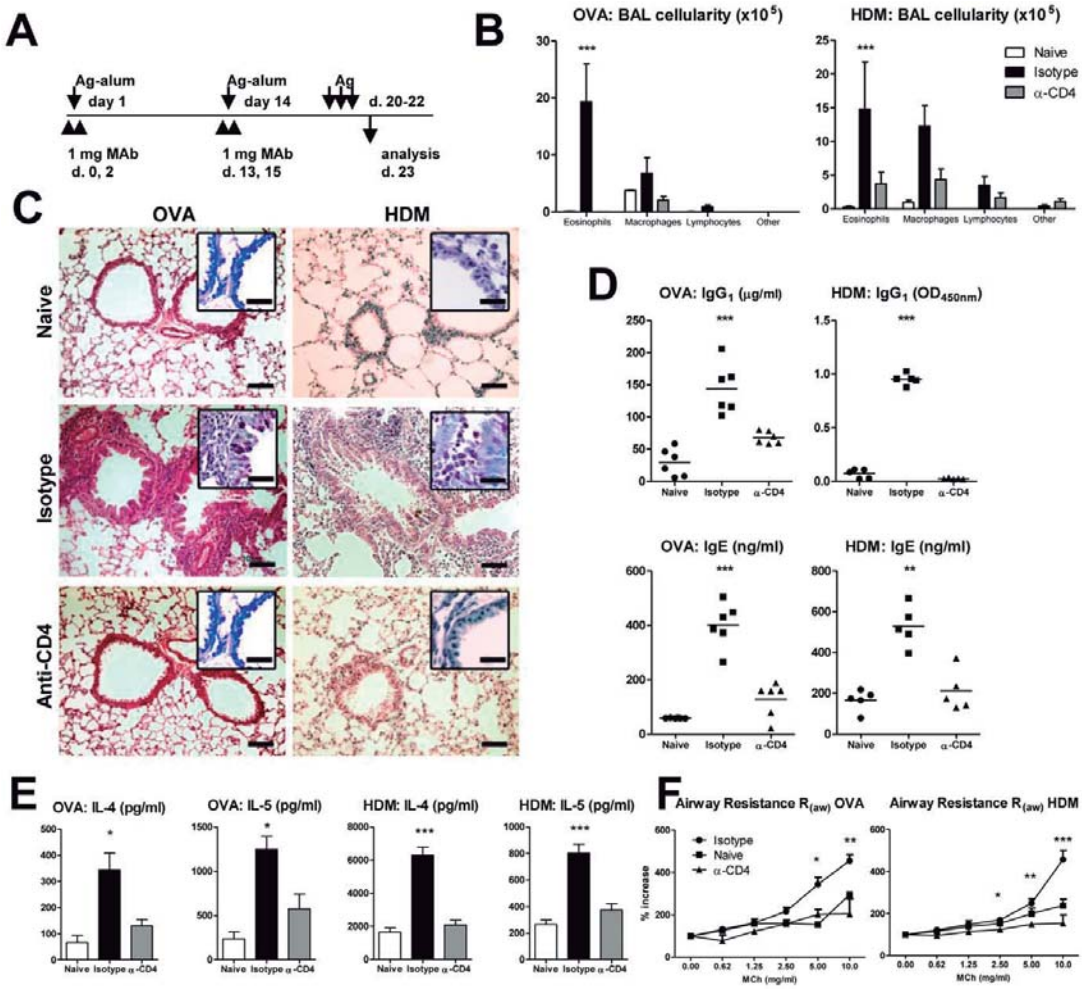

Figure 1. Prevention of allergic sensitization with anti-CD4 MAb. (A) Female BALB/c mice were sensitized with 20 μg OVA-alum or 50 μg HDM-Alum i.p. and challenged with 50 μg OVA or HDM in saline i.n. on the indicated days. Some animals were treated with 1 mg anti-CD4 or an isotype control i.p. as shown. Naive mice, not subjected to any intervention, were also studied as a control group. (B) Cellular composition of the BAL. Animals treated with anti-CD4 have less eosinophils in the BAL (n = 6, *** $P<0.001$). (C) Histological sections of lung tissue were stained with hematoxilin/eosin and PAS (inset). Anti-CD4-treated mice have reduced inflammatory infiltrate and goblet cell hyperplasia, to levels similar to naive controls. Bars represent 10 μm (2.5 μm in the inset). (D) Quantification of serum OVA- and HDM-specific IgG1 and IgE. Anti-CD4 MAb treated mice show a significant reduction of the Th2-driven immunoglobulins (n = 6, *** $P<0.001$ and ** $P<0.01$ as indicated). (E) The Th2 cytokines IL-4 and IL-5 were down to basal levels in lung homogenates of anti-CD4 treated mice (n = 6, * $P<0.05$ and *** $P<0.001$). (F) Invasive measurement of respiratory mechanics shows that animals treated with anti-CD4 MAbs had reduced airway resistance to increasing doses of inhaled MCh, when compared with sensitized control animals (n = 8). Data (B–F) are representative of at least three independent experiments.
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on the days before and after each immunization, and sacrificed 24 hours following the last intranasal challenge.

Mice treated with anti-CD4 had a marked reduction in BAL eosinophils when compared with sensitized animals, to levels similar to naïve animals or animals sensitized in the absence of the antigen (Figure 1B, and Figure S1). The absence of goblet cell hyperplasia and inflammatory infiltrate in the airways of anti-CD4 treated mice was confirmed by histology (Figure 1C). Furthermore, anti-CD4 treatment prevented effective generation of Th2-driven OVA- and HDM-specific IgG1 and IgE (Figure 1D). We could not detect Th1-driven antigen-specific IgG2a in any animal (not shown). Animals treated with anti-CD4 showed a marked reduction of IL-4 and IL-5 in lung homogenates to levels similar to naive animals (Figure 1E). Importantly, we found no evidence for Th1 or Th17 deviation (as inferred by levels of IFNγ or IL-17), nor increased levels of the immune-regulatory cytokine IL-10 (not shown). Cytokines in BAL were similar to lung homogenates (not shown).

To study the functional impact of the treatment we assessed AHR in response to increasing doses of inhaled MCh. Our data show that anti-CD4 treatment prevented AHR (Figure 1F and Figure S2).

The tolerance state is maintained following clearance of the MAb

We then studied whether tolerance induction with non-depleting anti-CD4 would protect the animals from subsequent exposure to the same antigens, at a time the therapeutic MAb had been cleared. For this purpose treated mice with anti-CD4 at the time of initial sensitization with OVA or HDM, and the same mice were again immunized with the same antigens 50 days following the initial treatment (Figure 2A).

Sensitization with OVA-alum or HDM-alum did not lead to airways eosinophilia in mice previously exposed to the same antigens under the cover of non-depleting anti-CD4 (Figure 2B and Figure S1). Furthermore, the treated mice also failed to produce antigen-specific IgG1 and IgE to OVA-alum and HDM-alum (Figure 2C). And importantly, AHR to increased concentrations of inhaled MCh was also absent in anti-CD4 treated mice (Figure 2D and Figure S2).

We could therefore conclude that a short course of anti-CD4 was leading to long-term effects. Although the MAb we used (clone YTS177) is known to have a non-depleting isotype [18], and we confirmed anti-CD4 treatment was not directly leading to T cell lysis (not shown), we had to confirm the treated mice remained immune competent.

Tolerant mice remain immunocompetent

In order to study the antigen-specificity of tolerance induction, we used a second unrelated antigen: β-lactoglobulin (β-LG). We compared immune responses to OVA and β-LG, since these are two defined antigens with similar characteristics, while HDM is a complex protein extract containing many distinct antigens.

BALB/c mice were treated with non-depleting anti-CD4 MAb together with OVA-alum (tOVA) or β-LG-alum (tβ-LG) in order to establish immune tolerance to those antigens (Figure 3A). At day 50 the animals were immunized with the same antigen used at the time of tolerization (day 0) or with the second unrelated antigen. All mice were subsequently challenged i.n. with the same antigen used at day 50. All animals remained protected from mounting airways inflammation in response to the antigen used for tolerization, but fully competent to undergo a Th2 response to the second antigen leading to airways eosinophilia, production of IgE and IgG1, and AHR (Figure 3B–D and Figure S2). These results suggest that CD4-blockade affects specifically the T cells that are being activated at the time of treatment, and sparing non-activated cells, thus leading to antigen specific tolerance where immune responses against different antigens are not suppressed.

Tolerance can be achieved in sensitized mice

To assess whether tolerance can be induced in pre-sensitized mice, BALB/c mice sensitized with OVA or HDM were treated with the same antigen under the cover of anti-CD4 50 days following initial sensitization (Figure 4A). For consistency with previous experiments we maintained the tolerance-inducing regime as two administrations of antigen-alum+anti-CD4 two weeks apart.

We found, both OVA- and HDM-sensitized mice treated with anti-CD4 were prevented from AHR, maintaining normal airway response to increased concentrations of inhaled MCh (Figure 4B and Figure S2). We confirmed the efficient sensitization of all groups of immunized animals by the presence of antigen-specific IgG1 and IgE antibodies in sera (not shown), although the mice had not been exposed to prior airway inflammation. As a consequence, the protection from AHR is effective in spite of high titres of antigen-specific immunoglobulins – possibly representing an impact on the late-phase response, and dissociation between high IgE and AHR.

We then assessed whether tolerance induction in pre-sensitized mice remained antigen-specific. BALB/c mice sensitized with OVA-alum or β-LG-alum were treated with anti-CD4 MAb, 50 days following the initial intervention, in the presence of either the initial (OVA or β-LG) or a different antigen (β-LG or OVA, respectively; Figure 4C). Sensitized mice were protected from airway eosinophilia when treated with anti-CD4 in the presence of the same antigen used for sensitization (OVA >tOVA and β-LG >tβ-LG, Figure 4D). The protective effect was, therefore, not due to the persistence of the therapeutic antibody in circulation at the time of intranasal exposure to the antigen since mice immunized with a different antigens not present during anti-CD4 treatment (and therefore with equivalent doses of circulating anti-CD4 at the time of challenge) were not protected (OVA >tβ-LG and β-LG >tOVA). The observation that animals receiving the antibody treatment together with a different antigen than used for immunization develop inflammatory changes similar to untreated control animals, or animals exposed to alum in the absence of the antigen (Figure S1), are consistent with the antigen-specificity of the tolerance state described above.

Mice exposed to allergic airways disease can be protected from AHR following anti-CD4 treatment

However, it is understood that the onset of inflammation in the airways becomes a significant hurdle for immune modulation leading to tolerance. Therefore, we investigated whether mice sensitized to OVA or HDM and exposed to the antigen i.n. could benefit from subsequent anti-CD4 treatment (Figure 5A). A single i.n. challenge with antigen in sensitized mice was sufficient to induce AHR (Figure S3).

We found that administration of HDM under the cover of non-depleting anti-CD4 30 days following induction of allergic airways disease was effective in preventing AHR following subsequent challenge with the same antigen (Figure 5B). We repeated the same studies with OVA with similar results (Figure 5B and Figure S2).

Discussion

Our data shows that CD4 blockade is effective in inducing antigen-specific tolerance to a clinically relevant allergen (HDM), thus preventing the manifestations of allergic airways disease following intranasal allergen challenge: Th2 and eosinophilic infiltrate of the airways, goblet cell hyperplasia, and AHR. The tolerogenic treatment is not only effective in preventing the disease in naive animals, but also confers considerable protection to mice previously sensitized with the allergen. Although in different experiments we tested mice with different ages, we could not find an age-related difference in their response to induction of allergic airways disease (Figure S4).

It should be noted that the use of tolerogenic MAbs in transplantation, with the same objective of preventing an inflammatory response to non-self antigens, have resulted in a different outcome from what we have observed. In transplantation, tolerance induction in sensitized animals has been difficult to achieve, except when both anti-CD4 and anti-CD8 MAbs are combined [19], with anti-CD8 MAbs probably required to control

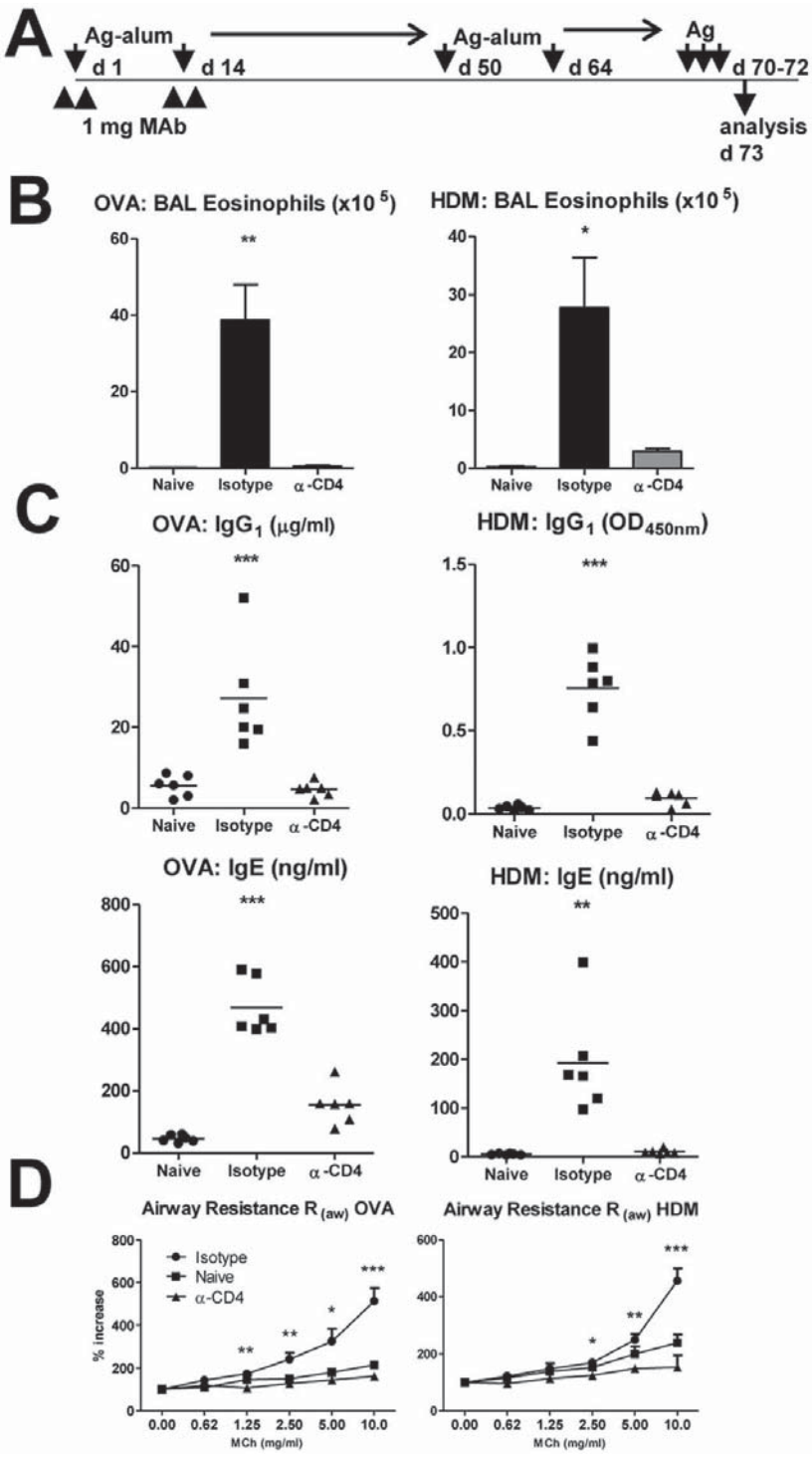
A
Ag-alum
d 1
d 14
Ag-alum
d 50
d 64
Ag
d 70-72
1 mg MAb
analysis
d 73
B
OVA: BAL Eosinophils (x10 5)
HDM: BAL Eosinophils (x10 5)
Naive
Isotype
α-CD4
C
OVA: IgG1 (μg/ml)
HDM: IgG1 (OD450nm)
OVA: IgE (ng/ml)
HDM: IgE (ng/ml)
D
Airway Resistance R(aw) OVA
Airway Resistance R(aw) HDM
Isotype
Naive
α-CD4
% increase
MCh (mg/ml)

Figure 2. Tolerized mice resist subsequent sensitization and challenge. (A) BALB/c mice were initially sensitized with OVA or HDM under the cover of anti-CD4 as described in Figure 1. Those mice were sensitized with the same antigens on days 50 and 64, and subsequently challenged i.n. (B) Animals trated with anti-CD4 were protected from BAL eosinophilia (n = 6, ** $P<0.01$ for OVA; * $P<0.05$ for HDM). (C) CD4-blockade prevented production of IgG1 and IgE in subsequent sensitizations (n = 6, *** $P<0.001$ and ** $P<0.01$ as indicated). (D) Tolerance to OVA or HDM prevented AHR to inhaled MCh (n = 6). Data are representative of three independent experiments.
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pre-committed effector T cells. Co-stimulation blockade with anti-CD40L has also been reported as less effective than anti-CD4, requiring depletion of $CD8^+$ T cells even in non-sensitized animals [20,21]. The obstacle created by sensitization in relation to tolerance induction is also evidenced by reports showing that heterologous immunity reduces the effectiveness of tolerogenic protocols in transplantation [22]. Our data shows that the anti-CD4 antibody treatment can also be beneficial to already sensitized animals. This may be due to the fact that the T effector cell frequency is significantly lower for allergens than for alloantigens and that the response to allergens is predominantly restricted to the $CD4^+$ compartment. It is likely that such different outcome observed in transplantation versus allergy may be due to the induction of different tolerogenic mechanisms. In fact our preliminary data suggests that $Foxp3^+$ Treg cells may play a more important role in transplantation tolerance than in tolerance induced to allergens – something that still requires further elucidation.

Our data also suggests it is likely that in sensitized mice the antibody treatment has an impact exclusively on the late response (mediated by Th2 and NKT cells), without preventing the early response mediated by mast cell degranulation in response to their surface IgE cross-linking, as the allergen-specific IgG1 and IgE titres remain high in treated mice. But even without targeting early mast cell degranulation, the MAb treatment is likely to lead to a putative long-term benefit given the importance of the Th2-mediated response for the persistence of the inflammatory changes associated with airways remodeling and chronic manifestations of the disease [4]. This issue will require confirmation in chronic models of disease.

The antigen-specificity of Treg cell-mediated tolerance has been a controversial issue [23,24]. We show that effective tolerance induction requires the presence of the appropriate antigen at the time of CD4 blockade leading to antigen-specific tolerance. In these experiments we waited 50 days following initial sensitization to minimize the amount of antigen still present in the animal at the time of the tolerogenic treatment. These data also established that MAbs administered at day 50 (Figure 3) were not contributing significantly to the prevention of the disease by being in circulation at the time of intranasal challenge, since no beneficial effect is observed in animals treated with the same antibody dose together with an irrelevant antigen. Importantly, most previous studies addressing the putative tolerance-inducing potential of monoclonal antibodies, namely anti-CD4 [15], did not address the antigen-specificity of the phenomenon or the immune competence of treated mice.

Several MAbs have been recently used as immune modulators in a wide range of diseases, including allergy [25]. Anti-CD4 MAbs have been evaluated both in pre-clinical non-human primate models of transplantation and autoimmunity, as well as in clinical studies [13,26,27]. Their therapeutic effectiveness was modest, short-term, and likely to be a consequence of transient immunosuppression and not tolerance. With hindsight those unimpressive results are not surprising due to technical details related with dosing and the MAb characteristics. The clinical trials have used mouse or chimeric MAb that elicited immune responses leading to their rapid clearance [28]. In addition, most of those studies, including a clinical trial in human asthma (with the depleting anti-CD4 MAb keliximab) [13], did not take advantage of non-depleting anti-CD4 MAbs. Therefore, and as a consequence of the adverse side effects associated with depleting reagents, it was not possible to attain a neutralizing dose of anti-CD4 know to be the most effective for tolerance induction. At this time, particularly given the promising results with non-depleting anti-CD3 in early onset diabetes patients [29], the past experience of anti-CD4 in human patients should be reassessed in face of

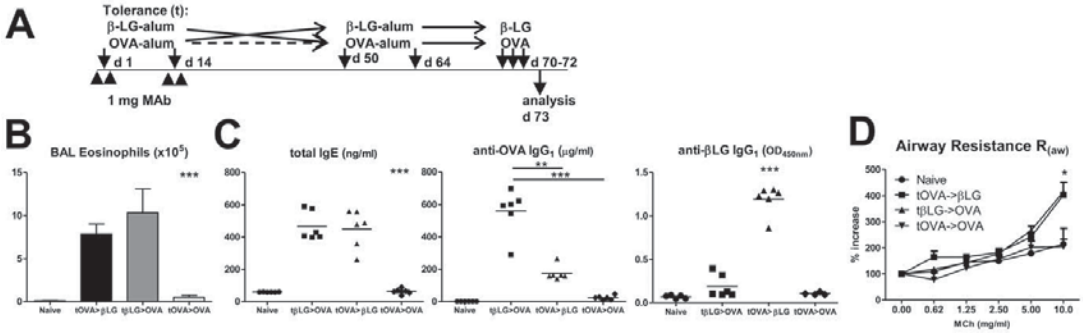

Figure 3. Mice treated with anti-CD4 are competent to respond to unrelated antigens. (A) Mice initially tolerized to OVA or β-LG (as described in previous figures) were sensitized i.p. with a different antigen at days 50 and 64, and challenged i.n. with the same antigen used at day 50. (B) Only animals tolerized to the same antigen used for sensitization at day 50 were protected from BAL eosinophilia (n = 6, *** $P<0.001$). (C) Tolerance induction to OVA did not prevent subsequent production of β-LG-driven IgE or IgG1, conversely, tolerance to β-LG did not hamper the generation of OVA-specific IgG1 or IgE (n = 6, *** $P<0.001$). (D) AHR in response to MCh was observed in animals tolerized to an antigen different from the one used for subsequent sensitization (tOVA >β-LG and tβ-LG >OVA) (n = 8, * $P<0.05$ at 10 mg/ml MCh). Data are representative of two independent experiments.
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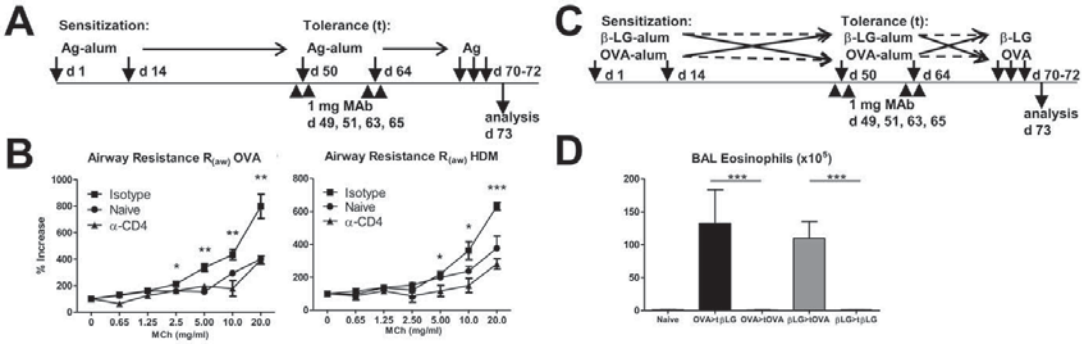

Figure 4. Tolerogenic effect of anti-CD4 treatment is antigen-specific and effective in sensitized animals. (A) BALB/c mice sensitized with OVA-alum or HDM-alum were tolerized to the same antigens on days 50 and 64, and challenged i.n. with the same antigens. (B) Sensitized mice subsequently treated with OVA or HDM under the cover of anti-CD4 showed protection from AHR (n = 8 for OVA, n = 6 to HDM). Data are representative of two independent experiments. (C) Mice were initially sensitized with OVA-alum or β-LG-alum, and tolerized to the same or a different antigen on days 50 and 64. All mice were challenged i.n. with the same antigen used for initial sensitization. (D) Mice treated with a different antigen together with anti-CD4 did not show reduced BAL eosinophilia (OVA >tβ-LG and β-LG >tOVA) while treatment with anti-CD4 and the same antigen used for sensitization showed a significant reduction of BAL eosinophilia (OVA>tOVA and β-LG >tβ-LG; n = 6, *** $P<0.001$). Data are representative of two independent experiments.
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current knowledge. On this note, a new non-depleting and less-immunogenic humanized anti-CD4 MAb has been recently engineered, and its safety evaluated in human volunteers [30].

Our results suggest the specific targeting of CD4 T cells in allergic airways disease can have a potent effect in achieving long-term protection from subsequent inflammatory changes induced

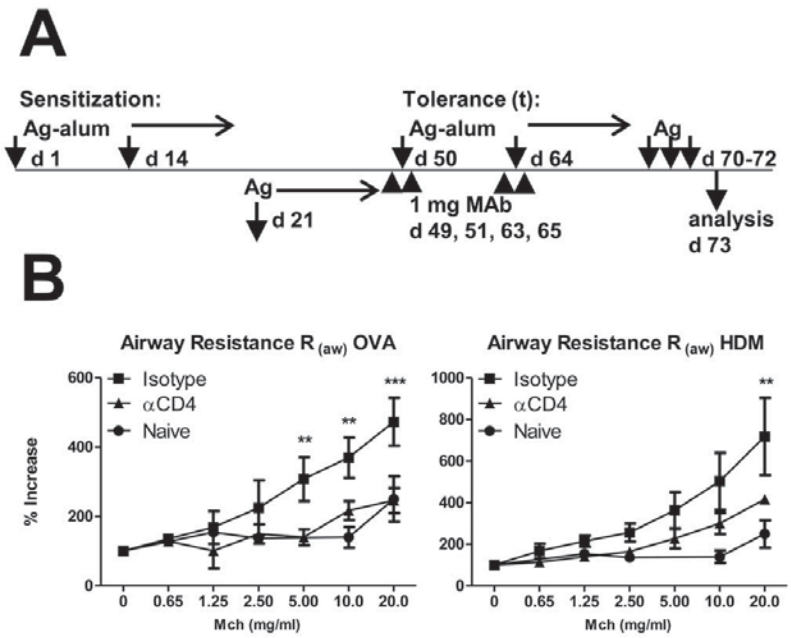

Figure 5. Protection from AHR in animals previously exposed to airways inflammation. (A) Balb/c mice sensitized and challenged i.n. with HDM-alum or OVA-alum were tolerized to the same antigens on days 50 and 64, and challenged i.n. with the same antigens. (B) The animals treated with HDM or OVA under the cover of anti-CD4 were protected from AHR in response to inhaled MCh (n = 6, ** $P<0.01$ or *** $P<0.001$ as indicated). Data are representative of two independent experiments.
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by the same antigens. It remains to be established whether similar effects can be achieved in chronic allergic airways disease.

Materials and Methods

Experimental animals

BALB/c mice were bred and maintained under specific pathogen-free facilities. Animals were sensitized, at the times described in the text, by i.p. injection of 20 μg in 2.0 mg of endotoxin-free aluminum hydroxide (Alu-gel-S, Serva, Heidelberg, Germany) of OVA or β-LG (Sigma, St Louis, USA) previously run through a DetoxyGel column (Pierce, Rockford, USA), or HDM extract (Greer, Lenoir, USA). In all experiments animals were age and sex matched.

Ethics Statement

All experiments involving animals were approved by Direccao Geral Veterinaria (approval 018831). Mice were bred and maintained under specific pathogen free (SPF) conditions.

Antibodies and reagents

Non-depleting anti-CD4 (YTS177) [18] and the isotype control (YKIX302) MAbs were produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland), and purified from culture supernatants by 50% ammonium sulfate precipitation, dialyzed against PBS, and the purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Bronchoalveolar Lavage (BAL)

Airways were washed through the trachea with 3 ml of cold PBS 1% BSA (Sigma). The BAL was centrifuged, ressuspended in PBS, and the cells counted with a hemocytometer. Differential cell counts were performed on cytospin samples stained with Giemsa-Wright (Sigma). At least 200 cells from each sample were counted, using blinded slides, to determine the relative frequency of each cell type. In addition, in some experiments eosinophilia was independently confirmed by flow cytometry using GR-1 (eBiosciences, San Diego, CA, USA), CCR3 (BD Pharmingen, San Diego, USA), and MHC-class II MAbs (produced in-house), with eosinophils identified based on the SSC/FSC profile and as the $GR1^{int}$MHCclass $II^{-}CCR3^{+}$ cells [31].

Quantification of immunoglobulins and cytokines

Serum titers of OVA-specific IgG1, IgG2a, and IgE were measured by ELISA using the following: IgG1 and IgG2a (SouthernBiotech, Birmingham, USA) with anti-OVA IgG1 standard from Serotec, Oxford, UK; IgE (BD-Pharmingen) with anti-OVA IgE standard from Abcam (Cambridge, UK). Cytokine titers were determined in fresh BAL and lung homogenates. Cytokine ELISAs were performed using the following kits: IL-4, IL-5 (BD-Pharmingen).

Histology

Lungs were perfused with 4% formalin solution (Sigma), collected and sectioned. Staining was performed using hematoxilin/eosin, and periodic acid-Schiff (PAS) stain. Photographs were taken using a Leica DM2500 microscope and a Leica DFC420 camera.

Respiratory mechanics and methacholine responsiveness

Airway responsiveness was determined 24 hours after last intranasal OVA challenge. Changes in the respiratory input impedance (Zrs) were measured using a modification of the low frequency forced-oscillation technique (LFOT) in mice anesthetized with 10 μl/g of xylazine (2 mg/ml, Ronpum, Bayer, Germany) and ketamine (40 mg/ml, Merial, Lyon, France), tracheostomized and ventilated (FlexiVent, SciReq, Montreal, Canada). Mice were hyperventilated at 450 breaths/min and Zrs was measured during periods of apnea using a 16 s signal containing 19 prime sinusoidal frequencies as described elsewhere [32]. Calculation of airway resistance (R_{aw}), tissue damping (G) and tissue elastance (H) is obtained from the Zrs spectrum using FlexiVent software (SciReq). AHR was measured by exposure to an aerosol containing increasing doses of MCh (Sigma), following a baseline measurement after the delivery of a saline aerosol.

Statistical analysis

Statistical significance was determined using the two-tailed non-parametric Mann-Whitney test and P values <0.05 were deemed significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Supporting Information

Figure S1 Prevention of allergic sensitization with anti-CD4 MAbs. Female BALB/c mice were sensitized with 20 μg OVA-alum i.p. and challenged with 50 μg OVA in saline i.n. on the indicated days. Some animals were treated with 1 mg anti-CD4 or an isotype control i.p. as shown. Naive mice, not subjected to any intervention, were also studied as a control group and compared with mice injected with adjuvant in the absence of antigen at the time of sensitization. (A) Cellular composition of the BAL of mice treated with anti-CD4 at the time of sensitization. (B) Cellular composition of the BAL of mice treated with anti-CD4 at the time of initial sensitization, but subjected to additional sensitization at a subsequent time. (C) Cellular composition of the BAL of sensitized mice treated with anti-CD4.
(TIF)

Figure S2 Invasive measurement of respiratory mechanics. Data showing the impact of anti-CD4 treatment in tissue elastance and tissue damping in response to increasing doses of inhaled MCh. These graphs complement the data on airway resistance represented in the main figures 1 to 5.
(TIF)

Figure S3 Induction of AHR following i.n. exposure to the antigen. Female BALB/c mice were sensitized with two shots of 20 μg OVA-alum i.p. 14 days apart, and challenged with 50 μg OVA in saline i.n. for three consecutive days (day 20–22), or just on day 20. Invasive measurement of respiratory mechanics was performed on the following day in presence of increasing doses of inhaled Mch. Both groups of mice, subjected to a single or three challenges with i.n. antigen, displayed similar levels of AHR ($n = 6$, ** $P<0.01$, *** $P<0.001$).
(TIF)

Figure S4 Allergic airways disease in mice with different age. Female BALB/c mice were sensitized with 20 μg OVA-alum i.p. and challenged with 50 μg OVA in saline i.n. as indicated in Figure 1. Some animals were treated with 1 mg anti-CD4 or an isotype control i.p. at the time of sensitization. Mice with 11 or 20 weeks of age were used. No significant differences between mice of different ages.
(TIF)

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Author Contributions

Conceived and designed the experiments: AA LG. Performed the experiments: AA. Analyzed the data: AA LG. Wrote the paper: AA LG.

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Monoclonal Anti-CD8 Therapy Induces Disease Amelioration in the K/BxN Mouse Model of Spontaneous Chronic Polyarthritis

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Objective. **CD8+ T cells are part of the T cell pool infiltrating the synovium in rheumatoid arthritis (RA). However, their role in the pathogenesis of RA has not been fully delineated. Using the K/BxN mouse model of spontaneous chronic arthritis, which shares many similarities with RA, we studied the potential of CD8+ T cell depletion with monoclonal antibodies (mAb) to stop and reverse the progression of experimental arthritis.**

Methods. **CD8+ T cells from the blood and articular infiltrate of K/BxN mice were characterized for cell surface phenotypic markers and for cytokine production. Additionally, mice were treated with specific anti-CD8 mAb (YTS105 and YTS169.4), with and without thymectomy.**

Results. **CD8+ T cells from the peripheral blood and joints of K/BxN mice were mainly CD69+ and CD62L−CD27+ T cells expressing proinflammatory cytokines (interferon-γ [IFNγ], tumor necrosis factor α [TNFα], interleukin-17a [IL-17A], and IL-4), and granzyme B. In mice receiving anti-CD8 mAb, the arthritis score improved 5 days after treatment. Recovery of the CD8+ T cells was associated with a new increase in the arthritis score after 20 days. In thymectomized and anti-CD8 mAb–treated mice, the arthritis score improved permanently. Histologic analysis showed an absence of inflammatory infiltrate in the anti-CD8 mAb–treated mice. In anti-CD8 mAb–treated mice, the serologic levels of TNFα, IFNγ, IL-6, and IL-5 normalized. The levels of the disease-related anti–glucose-6-phosphate isomerase antibodies did not change.**

Conclusion. **These results indicate that synovial activated effector CD8+ T cells locally synthesize proinflammatory cytokines (IFNγ, TNFα, IL-17, IL-6) and granzyme B in the arthritic joint, thus playing a pivotal role in maintaining chronic synovitis in the K/BxN mouse model of arthritis.**

Approximately 40% of the T cells infiltrating the rheumatoid synovial membrane are CD8+ T cells (1). However, their importance in the pathogenesis of rheumatoid arthritis (RA) remains to be fully elucidated.

The primary function of CD8+ T cells is the killing of virus- or cytosolic bacteria–infected cells. Moreover, they seem to play several important roles in autoimmune diseases, either protecting against or enhancing the disease. In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, CD8+ T cells have been shown to be crucial for resistance to a second induction of the disease (2). Recently, a particular subset of CD8+ T cells (CD8+CD122+) was shown to accelerate the recovery of animals with EAE after CD8+ T cells were trans-

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ferred (3). In contrast, insulitis failed to develop in NOD mice treated with anti-CD8 monoclonal antibodies (mAb) (4). This experimental treatment also inhibited the transfer of insulin-dependent diabetes mellitus (IDDM) and the development of spontaneous IDDM (5).

In RA, some patients show CD8+ T cell clonal expansions with a memory phenotype that are correlated with rheumatoid factor (RF) levels (6–10). This is most likely attributable to the important role of CD8+ T cells in maintaining ectopic germinal center structures in RA synovium (11,12).

In different animal models of collagen-induced arthritis (CIA), the absence of CD8+ T cells resulted in a reduced incidence (13,14) and severity (15) of the disease. However, higher susceptibility was observed when animals were rechallenged (14,15). Additionally, in $CD8^{+/-}$ and $CD8^{-/-}$ mice, a trend toward a delayed onset of CIA was observed, without a significant impact on disease susceptibility (16). More recently, CD8+ T cell clones generated from the arthritic joints of SKG mice transferred to histocompatible athymic nude mice led to joint swelling and synovitis with destruction of cartilage and bone (17).

In order to assess the role of CD8+ T cells in experimental chronic polyarthritis, the clinical phenotype and cytokine production of articular and peripheral blood CD8+ T cells from K/BxN mice were studied. Arthritis in these mice results from the simultaneous expression of the class II major histocompatibility complex A^{g7} molecule and a transgenic T cell receptor (TCR), followed by the production of autoantibodies against glucose-6-phosphate isomerase (GPI) (18–20). Subsequently, we assessed whether treatment with specific anti-CD8 mAb, with and without thymectomy, improved the course of established arthritis in K/BxN mice. Our results showed, for the first time, that K/BxN mouse–activated and effector memory CD8+ T cells are present in the peripheral blood and joints and that they play an important role in arthritis maintenance, because treatment with specific anti-CD8 mAb significantly improved the disease signs. These results document that CD8+ T cells should be regarded as major players in the K/BxN mouse model of experimental arthritis, along with CD4+ T cells and B cells.

MATERIALS AND METHODS

Mice. TCR-transgenic KRN mice were a kind gift from Dr. C. Benoist (Harvard University, Boston, MA) and were maintained on a C57BL/6 background (K/B). Progeny bearing the $V_\beta 6$-transgenic TCR were identified at 3–4 weeks of age by cytofluorometry of peripheral blood lymphocytes, using phycoerythrin (PE)–labeled anti-CD8 (clone YTS169.4; Instituto Gulbenkian de Ciência [IGC] Cell Imaging Unit) and fluorescein isothiocyanate (FITC)–labeled anti-$V_\beta 6$ (PharMingen) antibodies. Arthritic mice were obtained by crossing K/B mice with NOD mice (K/BxN). C57BL/6 and NOD mice were provided by the IGC Animal Facility. The mice were bred and maintained under standard conditions, with food and water available ad libitum, in a specific pathogen–free environment. All animal studies were approved by the internal IGC Ethics Committee.

Antibodies and immunization in mice with established arthritis. Depleting anti-CD8 (clone YTS169.4), nondepleting anti-CD8 (clone YTS105), and rat IgG2a isotype control (clone YKIX302) mAb were a kind donation from Prof. H. Waldmann (Oxford University, Oxford, UK). The aim of combining nondepleting YTS105 mAb (21) and depleting YTS169.4 mAb (22) was to reduce the immunogenic potential of the antibodies (and their subsequent neutralization) that could be created after repeated injections. Mice ages 8–10 weeks old with an arthritis score >8 were injected intraperitoneally with either 150 μg of nondepleting anti-CD8 (n = 20) or anti-dog IgG isotype control (n = 19) on day 0. A second and third dose of 150 μg of depleting anti-CD8 or anti-dog IgG isotype control antibodies were injected intraperitoneally on days 7 and 16 after the first injection.

Thymectomy and CD8 depletion. Five-week-old K/BxN mice with established arthritis were subjected to total thymectomy (n = 5) or a sham operation (n = 3). Nine days after surgery, the mice were immunized intraperitoneally with 300 μg of depleting anti-CD8 antibody.

Arthritis scoring. Each swollen fore paw or hind paw was given a score of 1 point, each swollen wrist or ankle was given a score of 1 point, and each swollen finger or toe was given a score of 0.5 point, resulting in a maximum of 17 points per mouse. Scoring was performed every second day for the first 3 weeks and then once weekly for the remaining observation period.

Histochemical analysis. Skinless whole knee joints and front and hind paws were fixed in 5% formalin, decalcified in 5% formic acid, and embedded in paraffin. Sections (10 μm) were prepared from the tissue blocks and stained with either hematoxylin and eosin (H&E), MNF116 (anticytokeratin antibody), Herovici's stain, or Alcian blue–periodic acid−Schiff and observed on an Olympus IMT-2 microscope. Images were analyzed with ImageJ 1.38x software (National Institutes of Health).

Enzyme-linked immunosorbent assay (ELISA) for GPI. High-affinity Maxisorb 96-well ELISA plates (Nunc) were coated with 10 n*M Saccharomyces cerevisiae* GPI (Sigma) in potassium phosphate buffer. Plates were blocked with phosphate buffered saline/Tween/1% gelatin. Anti-GPI antibodies in sera were detected with horseradish peroxidase–labeled goat anti-mouse IgG (Southern Biotechnology) followed by incubation with tetramethylbenzidine solution (Sigma). Absorption was measured at an optical density of 450 nm.

Flow cytometric analysis. Peripheral blood samples were collected from the base of the tails of arthritic K/BxN mice on days 0, 7, 14, 21, and 35 after the first treatment with either anti-CD8 or control mAb. Mononuclear cells were isolated through a Ficoll gradient (Amersham) and stained

with anti-mouse mAb as follows: fluorescein isothiocyanate (FITC)–labeled anti-CD3 (clone 145.2C11), PE-labeled anti-CD8 (clone YTS169.4), and allophycocyanin (APC)–labeled anti-CD4 (clone GK1.5-8) (all from the IGC Cell Imaging Unit). To determine the differences in CXCR4 and CXCR5 expression and the frequency of CD8+ T cell subsets, peripheral blood samples were collected from the tails of 8–10-week-old untreated arthritic K/BxN mice (n = 9) and their nonarthritic littermates (n = 10). Mononuclear cells were isolated through a Ficoll gradient (Amersham) and stained with anti-mouse mAb, as follows: FITC-labeled anti-CD19 (clone 1D3; IGC Cell Imaging Unit), PE-labeled anti-CXCR4 (eBioscience), peridinin chlorophyll A protein (PerCP)–labeled anti-CD4 (eBioscience), APC-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit), FITC-labeled anti-CD3 (clone 145.2C11; IGC Cell Imaging Unit), PE-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit), biotinylated anti-CD62 ligand plus streptavidin–PerCP, and APC-labeled anti-CD27 (all from eBioscience). For determination of intracellular cytokine production, saponin-permeabilized peripheral blood mononuclear cells (PBMCs) were stained with APC-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit), PerCP-Cy5.5–labeled anti-CD3, FITC-labeled anti-TNFα, and PE-labeled anti–IL-6 (all from eBioscience). All samples were analyzed on a 4-color FACSCalibur system (Becton Dickinson), and data were analyzed with FlowJo 7.5.5 software (Tree Star).

Assessment of intracellular cytokine production by reverse transcription–polymerase chain reaction (RT-PCR). PBMCs and mononuclear cells from articular tissue were collected from untreated arthritic mice (n = 4), and PBMCs were collected from their healthy control littermates (n = 4). CD8+ T cells were isolated by magnetic cell separation using the CD8a+ T Cell Isolation kit (Miltenyi Biotech).

Total RNA from sorted CD8+ T cells was isolated using an RNeasy Micro kit (Qiagen). RNA integrity and quantification were analyzed using a 6000 Nano Chip kit in an Agilent 2100 Bioanalyzer. RNA was reverse transcribed with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo(dT) plus random hexamers according to the manufacturer's instructions.

Relative quantification of gene expression by real-time PCR was performed using a thermocycler LightCycler 480 II (Roche). Normalization for gene expression quantification was performed with geNorm Housekeeping Gene Selection Mouse kit (PrimerDesign) and geNorm software (Ghent University Hospital, Center for Medical Genetics) to select optimal reference genes for this study (23).

Real-time PCRs used specific *Mus musculus* QuantiTect Primer Assays (Qiagen) with optimized primers for the genes of interest, *Gzmb* (QT00114590), *Ifn* (QT01038821), *Il10* (QT00106169), *Il17a* (QT00103278), *Il2* (QT00112315), *Il4* (QT00160678), *Tnf* (QT00104006), and the reference genes *Ywhaz* (QT00105350) and *Rn18s* (QT01036875), together with a QuantiTect SYBR Green PCR Gene Expression kit (Qiagen), according to the manufacturer's instructions. Reactions were performed with the following thermal profile: 10 minutes at 95°C plus 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Quantitative real-time PCR results were analyzed using LightCycler 480 software (Roche) and quantified using the qBasePlus software package (Biogazelle).

Serum cytokine quantification. Serum samples from arthritic K/BxN mice before (n = 11) and after (n = 11) anti-CD8 treatment and from their nonarthritic control littermates (n = 7) were obtained from whole blood after centrifugation. TNFα, IFNγ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and monocyte chemoattractant protein 1 (MCP-1) titers in the sera were quantified using the cytometric bead arrays, a Mouse Th1/Th2 Cytokine kit and a Mouse Inflammation kit (Becton Dickinson) according to the manufacturer's instructions, and analyzed with BD Cytometric Bead Array Software (Becton Dickinson). IL-17a titers were determined using a Mouse IL-17A ELISA kit (Invitrogen). Test sensitivity thresholds for the different cytokines were as follows: for TNFα, 6.3 pg/ml; for IFNγ, 2.5 pg/ml; for IL-2, 5.0 pg/ml; for IL-4, 5.0 pg/ml; for IL-5, 5.0 pg/ml; for IL-6, 5.0 pg/ml; for IL-10, 17.5 pg/ml; for IL-12p70, 10.7 pg/ml; for IL-17a, 5.0 pg/ml; and for MCP-1, 52.7 pg/ml. Mean titers below those thresholds were considered undetectable.

Statistical analysis. Data were checked for normality, in order to decide whether to use the parametric one-way analysis of variance and post hoc Tukey's test or the nonparametric Kolmogorov-Smirnov test. Data were analyzed using StatView 5.0 software (Abacus Concepts). *P* values less than 0.05 were considered significant.

RESULTS

Activation of K/BxN mouse CD8+ T cells in the articular infiltrate. In an effort to characterize the CD8+ T cells in K/BxN mice, mononuclear cells were isolated from the peripheral blood and from the articular inflammatory infiltrate and analyzed for the expression of surface markers and cytokine production. In contrast to the circulating CD4+ T cell pool, a significantly higher ($P < 0.05$) percentage of circulating CD8+ T cells from K/BxN mice expressed the $V_\beta 6$-transgenic TCR (mean ± SD 32 ± 10% and 84 ± 7% for CD4 and CD8, respectively) at 3 weeks after birth, before any external clinical signs of arthritis could be detected.

The frequencies of CD8+ T cell subsets defined by the expression of CD27 and CD62L in the peripheral blood and articular infiltrate from arthritic K/BxN mice were compared with those in the peripheral blood of healthy mice (Figure 1A). The frequency of CD27−CD62L− short-lived effector CD8+ T cells (T_{se}) was similar in both K/BxN mouse tissue and peripheral blood from healthy mice. However, the peripheral blood of arthritic K/BxN mice presented a significantly ($P = 0.019$) higher frequency of CD27+CD62L− effector memory CD8+ T cells (T_{em}) than the peripheral blood of healthy mice. Moreover, the frequency of this T_{em} subset was higher, although not reaching statistical significance ($P = 0.0791$), in the articular infiltrate than in the peripheral blood of K/BxN mice. The frequency of CD27+CD62L+ central memory CD8+ T cells (T_{cm}) was comparable in the peripheral blood of K/BxN mice

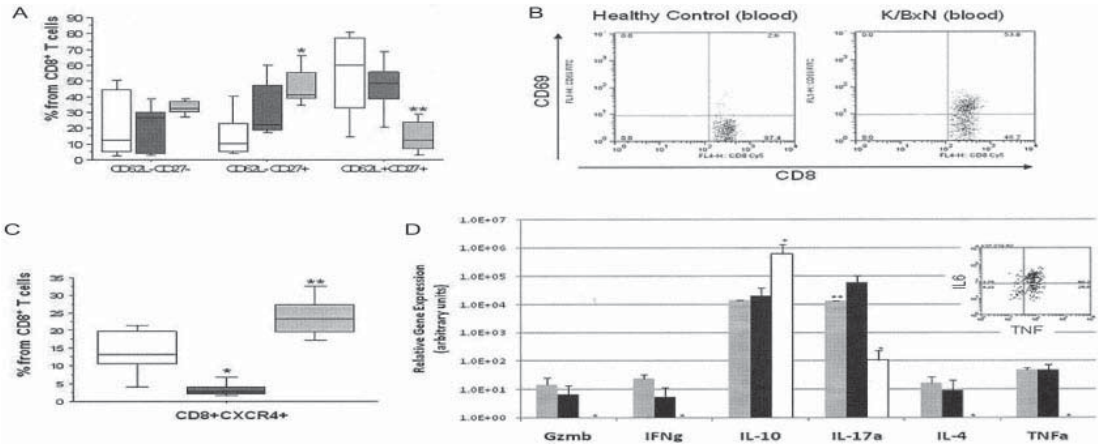

Figure 1. CD8+ T cells of K/BxN mice present an activated effector memory phenotype, homing preferentially to the articular tissue where they produce proinflammatory cytokines. **A** and **C,** Frequency of CD62L−CD27−, CD62L+CD27+, and CD62L−CD27+ CD8+ T cells (**A**) and frequency of CD8+CXCR4+ CD8+ T cells in the blood of healthy control mice (open boxes; n = 10), the blood of K/BxN mice (darkly shaded boxes; n = 9), and the articular tissue of arthritic K/BxN mice (lightly shaded boxes; n = 9). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. * = $P < 0.05$ versus control; ** = $P < 0.05$ versus K/BxN mouse blood. **B,** Dot plots of CD69 versus CD8 in the blood of a representative healthy control mouse and an arthritic K/BxN mouse. **D,** Relative expression of several cytokine genes in unstimulated CD8+ T cells isolated from the articular tissue (lightly shaded bars; n = 4) and peripheral blood (darkly shaded bars; n = 4) of arthritic K/BxN mice and from control peripheral blood (open bars; n = 4). Bars show the mean and SD. **Inset,** Intracellular production of tumor necrosis factor α (TNF) and interleukin-6 (IL-6) in CD8+ T cells from the articular tissue of a representative K/BxN mouse. * = $P < 0.01$ versus K/BxN mouse blood and articular tissue; ** = $P < 0.05$ versus K/BxN mouse blood. IFNγ = interferon-γ; Gzmb = granzyme B.

and that of healthy mice. However, the frequency of T_{cm} was significantly lower ($P = 0.008$) in the articular infiltrate of K/BxN mice than in the peripheral blood of K/BxN mice.

In contrast to what was observed in healthy mice, the majority of CD8+ T cells circulating in K/BxN mouse peripheral blood expressed the early activation marker CD69 (Figure 1B), and this increased expression of CD69 was also observed on the surface of CD8+ T cells infiltrating the joints.

To assess whether the expression of chemokine receptors by K/BxN mouse articular CD8+ T cells could contribute to the skewed distribution of the different CD8+ T cell subsets in the articular infiltrate, the frequencies of CD8+ T cells expressing specific chemokine receptors were determined (Figure 1C). Interestingly, the frequency of CD8+CXCR4+ was significantly increased in the articular tissue when compared with the peripheral blood ($P = 0.002$) of K/BxN mice. Moreover, the peripheral blood of K/BxN mice had a significantly ($P = 0.0007$) decreased frequency of CD8+CXCR4+ T cells when compared with that of controls. In contrast to what has been reported in humans (24), we were not able to clearly identify a circulating CXCR5-expressing CD8+ T cell population in either the healthy mice or the K/BxN mice (data not shown).

To determine whether CD8+ T cells infiltrating the joints of arthritic K/BxN mice had the potential to actively participate in the inflammatory and joint destruction process by producing proinflammatory cytokines and cytolytic enzymes, we quantified the relative gene expression of several cytokines and granzyme B in unstimulated CD8+ T cells. As depicted in Figure 1D, both articular tissue and peripheral blood CD8+ T cells from arthritic K/BxN mice had similar expression of the genes coding for granzyme B, IFNγ, IL-4, and TNFα, while no expression of these genes was detected in CD8+ T cells isolated from control peripheral blood. Interestingly, more than half of the articular CD8+ T cells producing TNFα also produced IL-6 (inset in Figure 1D). However, expression of the gene coding for IL-17a was significantly higher ($P < 0.01$) in K/BxN mouse peripheral blood CD8+ T cells than in articular tissue or control peripheral blood. Nevertheless, the CD8+ T cells from the articular tissue still expressed significantly higher levels of *Il17a* than did the control

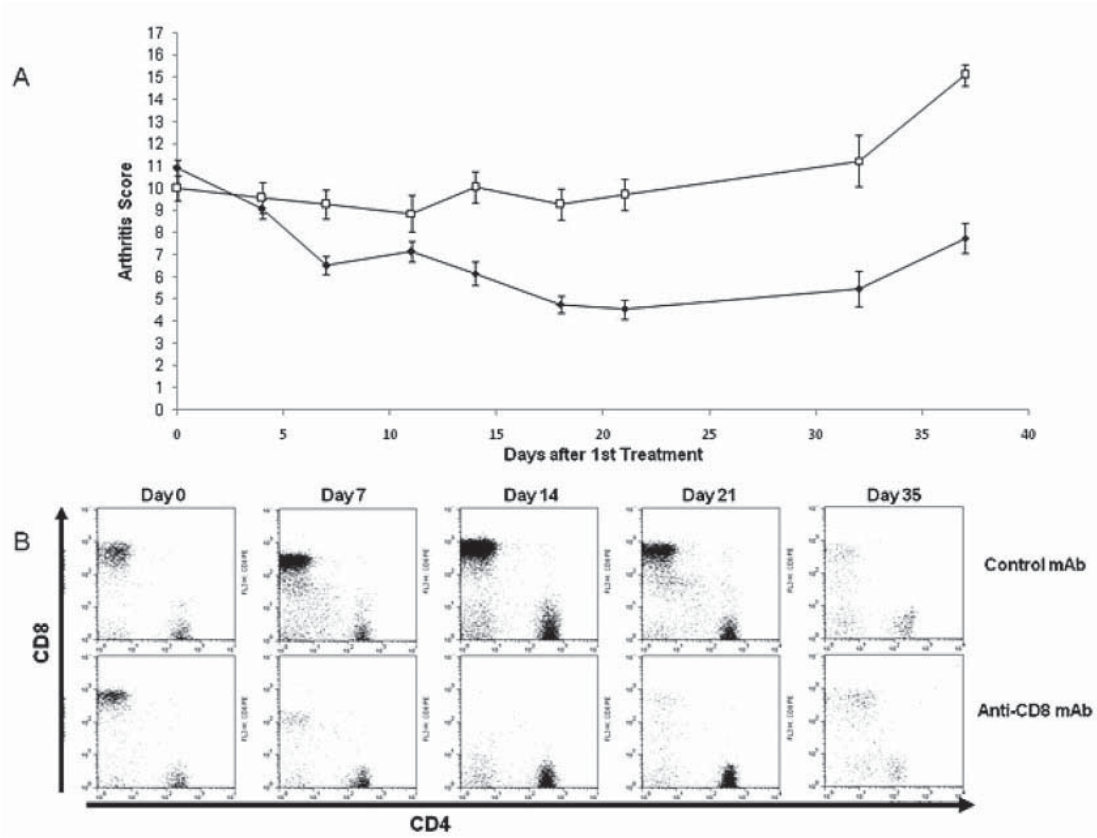

Figure 2. Treatment with anti-CD8 monoclonal antibodies (mAb) after polyarthritis is established ameliorates disease signs in K/BxN mice, and disease relapse occurs with CD8+ T cell recovery. **A,** Evolution of the disease score over 40 days in the control mAb–treated group (squares; n = 19) and the anti-CD8 mAb–treated group (diamonds; n = 20). Mice received an injection of YTS105 (blocking) or mock antibody on day 0 and an injection of YTS169.4 (depleting) or mock antibody on days 7 and 16. Values are the mean ± SEM. **B,** Representative dot plots of CD4 versus CD8 in CD3+ peripheral blood T cells on days 0, 7, 14, 21, and 35 after treatment with anti-CD8 mAb or control mAb.

peripheral blood. As expected, the expression of *Il10* was increased in the CD8+ T cells of all 3 tissue types, with the control peripheral blood presenting a significantly higher expression ($P < 0.05$), while no *Il2* gene expression was detected in any of the CD8+ T cells isolated from the different tissues.

Improvement in macroscopic and microscopic signs of disease by depletion of CD8+ T cells with mAb. To assess the importance of CD8+ T cells in the maintenance of chronic polyarticular inflammation in K/BxN mice, specific mAb that either blocked (YTS105) or depleted (YTS169.4) CD8+ T cells were administered after arthritis was established (arthritis score >9). As shown in Figure 2A, the arthritis scores for the mice treated with anti-CD8 mAb began to improve starting 5 days after the initial treatment, as compared with the groups receiving control mAb. Furthermore, a lower arthritis score was maintained for more than a month thereafter in the anti-CD8–treated group. The increase in the arthritis score in the anti-CD8–treated mice observed from day 21 onward corresponded to a recovery of the CD8+ T cell pool (Figure 2B).

Histologic analysis of the hind paw ankle joints revealed an absence of inflammatory infiltrate accompanied by new bone formation and normal synovial bursae in arthritic K/BxN mice 30 days after receiving

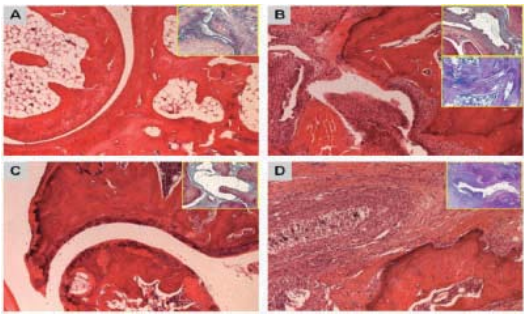

Figure 3. Histologic assessment of articular tissue shows clearance of the inflammatory infiltrate in anti-CD8 monoclonal antibody–treated K/BxN mice. **A,** Joint section from a healthy control mouse (H&E stained; original magnification × 10). **Inset,** Normal synovial bursae (Herovici stained; original magnification × 200). **B,** Joint section from a K/BxN mouse before treatment, showing massive inflammation and cartilage/bone destruction (H&E stained; original magnification × 10). **Upper inset,** Inflamed hyperplastic synovium (Herovici stained; original magnification × 100). **Lower inset,** Chondral sclerosis and fibrous ankylosis (Alcian blue–periodic acid−Schiff stained; original magnification × 100). **C,** Joint section from an anti-CD8 monoclonal antibody–treated K/BxN mouse 30 days after treatment, showing complete clearance of the inflammatory infiltrate and normalization of the articular architecture (H&E stained; original magnification × 10). **Inset,** Normal synovial bursae (Herovici stained; original magnification × 100). **D,** Joint section from a control monoclonal antibody–treated K/BxN mouse 30 days after treatment, showing complete destruction of the joint structure by massive infiltration of inflammatory cells and fibrosis (H&E stained; original magnification × 10). **Inset,** Proliferative synovitis with destruction of articular cartilage (Alcian blue–periodic acid–Schiff stained; original magnification × 100).

anti-CD8 mAb (Figure 3C), as opposed to mice receiving control mAb, which presented an inflamed hyperplastic synovium and articular erosions (Figure 3D).

To investigate whether the arthritis improvement after anti-CD8 therapy was associated with changes in the levels of circulating cytokines, the concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17a, MCP-1, TNFα, and IFNγ were measured in the serum of healthy control and K/BxN mice at baseline and 20 days after the initial treatment (Figures 4A–D). Arthritic K/BxN mice that were assessed before treatment had significantly higher ($P < 0.02$) serologic titers of IL-5, IL-6, and TNFα than healthy controls. Twenty days after anti-CD8 therapy, the serologic levels of all 3 cytokines and IFNγ had significantly dropped ($P < 0.04$) from their baseline values and were comparable with the ones present in healthy control mice. The serologic levels of all other cytokines did not pass the minimum test threshold.

Prevention of arthritis relapse by complete thymectomy followed by depletion of CD8+ T cells. In an effort to verify whether a permanent absence of CD8+ T cells could protect K/BxN mice from arthritis relapse, 5–6-week-old K/BxN mice with established polyarthritis (arthritis score >8) underwent total thymectomy followed by the injection of a high dose of depleting (YTS169.4) anti-CD8 mAb 9 days later. Control mice underwent sham operations and received an equal dose of depleting anti-CD8 mAb 9 days later, after which arthritis evolution was monitored for a further 90 days.

Thymectomy alone did not seem to induce a short-term alteration of the course of the disease, since no significant changes in the arthritis score could be observed between day 0 and day 9. Administration of anti-CD8 mAb led to an amelioration of the clinical signs of arthritis in both the thymectomized and control K/BxN mice. However, although the control mice had a relapse of the disease 43 days after having received the depleting anti-CD8 mAb (the longer time before relapse observed in these mice compared with those in Figure 2A is attributable to the higher dose of anti-CD8 mAb they received), the thymectomized mice experienced further arthritis improvement, which lasted until the end of the 90-day followup (Figure 5A). The absence of complete clinical remission in the thymectomized mice (arthritis score 0) is attributable to the effects of residual deformities on the scoring system. In fact, the histologic sections obtained on day 90 from the hind paws of the thymectomized mice showed an absence of inflammatory infiltrate in the synovial membrane and preservation of the articular and bone structure (Figure 5B), as opposed to the expanded inflammatory infiltration and extensive arthrosis observed in the sham-operated controls (Figure 5C).

Effect of disease amelioration on anti-GPI antibody titers. The development and maintenance of polyarthritis in K/BxN mice has been linked to the production of anti-GPI autoantibodies, the serum concentration of which increases with age and disease progression (20). Therefore, we determined the serologic titers of anti-GPI IgG in K/BxN mice at baseline (before mAb treatment or thymectomy was started) and after 30 days of YTS105 followed by YTS169.4 anti-CD8 treatment or control anti-dog IgG treatment. Similar assessments were performed 90 days after thymectomy or sham operation and YTS169.4 treatment in K/BxN mice and age-matched nonarthritic control littermates (treated either with YTS105 followed by YTS169.4 anti-CD8 or control anti-dog IgG), but no significant effects of treatment on the anti-GPI IgG titers were

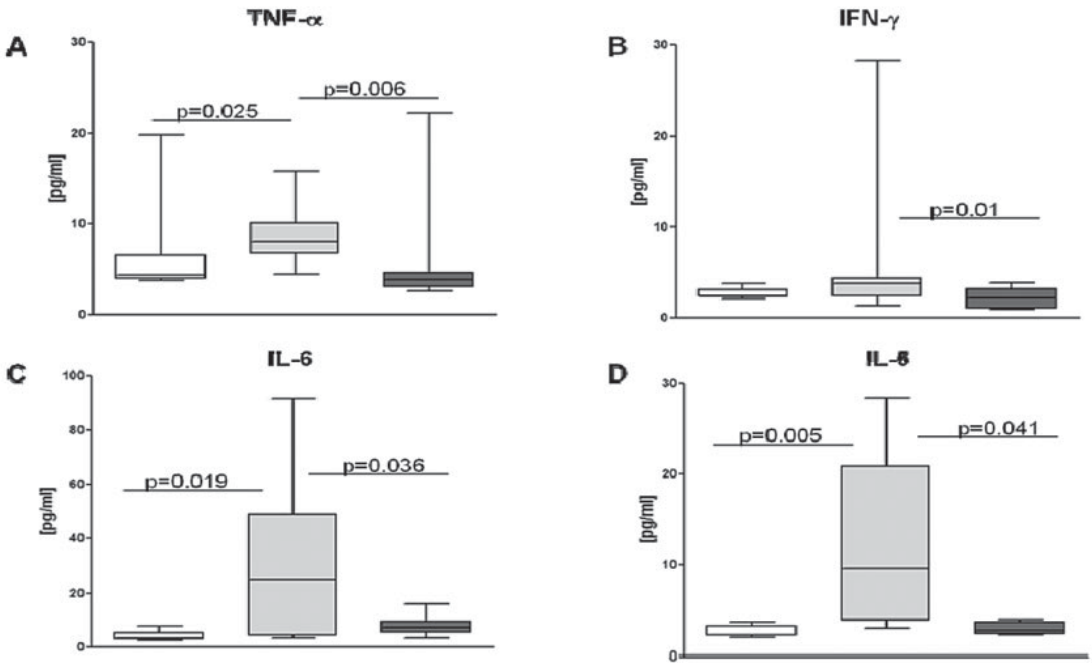

Figure 4. Treatment with anti-CD8 monoclonal antibodies normalizes the serologic levels of proinflammatory cytokines in K/BxN mice. Titers of tumor necrosis factor α (TNFα) (**A**), interferon-γ (IFNγ) (**B**), interleukin-6 (IL-6) (**C**), and IL-5 (**D**) in the blood of untreated K/BxN mice (lightly shaded boxes; n = 11), the blood of healthy control mice (open boxes; n = 7), and the blood of anti-CD8–treated K/BxN mice (darkly shaded boxes; n = 11) are shown. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles.

observed (Figure 6). No significant effects of treatment on anti-GPI IgG titers were observed (Figure 6). Actually, the titers of anti-GPI antibodies increased in the thymectomized mice even though inflammation of the joints subsided. The frequency of circulating CD138+ plasma cells was also not affected by any of the treatments (data not shown).

DISCUSSION

The role of CD8+ T cells in the pathogenesis of RA remains unclear. Nevertheless, several studies in patients with RA that associated the effector functions and the memory CD45RO+ and activated "false" memory CD29+CD45RA+CD45RO− phenotypes of CD8+ T cells with RF production and disease activity point out that the contribution of CD8+ T cells to RA should be reevaluated (6,7,9,10). This is also the case in animal models, because data from the literature on experimental arthritis are few and contradictory. Several studies on the CIA model of experimental polyarthritis focusing on the involvement of CD8+ T cells in initiating arthritis (13,14,25) revealed that anti-CD8 treatment rendered experimental animals less susceptible (14) or fully resistant (13) to the disease. Another study involving NOD/SCID mice engrafted with human rheumatoid synovium stressed the importance of CD8+ T cells for the maintenance of synovial follicular-like structures (11).

Most studies were carried out in the CIA model and manipulated the CD8+ T cell response before arthritis induction, thus focusing on the potential role of CD8+ cells in the initiation of the disease (13,14,16,25). This is consistent with current paradigms regarding the role of CD8+ cells but was also favored because of the transient nature of CIA, which would render it difficult to distinguish between the ameliorating effect of CD8 blockade and natural disease remission. Therefore, the contribution of CD8+ T cells to the chronicity of polyarthritis has not been addressed.

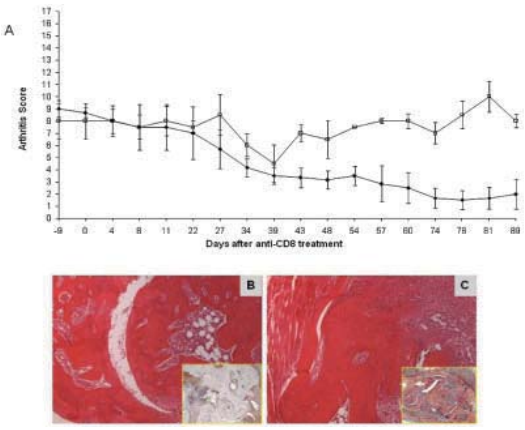

Figure 5. Thymectomy followed by CD8+ T cell depletion stops arthritis relapse, reduces the inflammatory infiltration of the joint, and preserves bone and articular integrity in K/BxN mice. **A,** Evolution of the arthritis score for 90 days after CD8+ T cell depletion in sham-operated control mice (squares; n = 3) and thymectomized mice (diamonds; n = 5). Surgery was performed on day −9, and CD8 depletion was performed on day 0. Values are the mean ± SEM. **B,** Section from the hind paw of a K/BxN mouse 90 days after thymectomy and CD8+ T cell depletion (H&E stained; original magnification × 10). **Inset,** Preserved joint and synovial proliferation without inflammation (MNF116 stained; original magnification × 100). **C,** Section from the hind paw of a K/BxN mouse 90 days after sham operation and CD8+ T cell depletion (H&E stained; original magnification ×10). **Inset,** Arthrosis of the joint (Herovici stained; original magnification × 40).

The recent development of murine models of persistent chronic polyarthritis—the SKG (26), the K/BxN (19), and the B10.Q/Ncf1* (27) models—provide new tools for studying CD8+ T cell involvement in arthritis maintenance.

In the present study, we used the K/BxN mouse model of chronic polyarthritis to show that CD8+ T cells circulating in the peripheral blood and infiltrating the joints are responsible for maintaining chronic articular inflammation. An evident decrease in articular swelling and redness a few days after the initial anti-CD8 treatment was confirmed by the absence of histologic signs of inflammatory infiltrates and evidence of de novo ossification. Moreover, normalization of the serologic levels of proinflammatory cytokines, such as TNFα, IFNγ, and IL-6, in the anti-CD8 mAb–treated mice represents evidence that the role of CD8+ T cells in arthritis maintenance is at least partially mediated through self-production of these cytokines or by (co)stimulation of production in other cells. Additionally, normalization of the serologic levels of IL-5, a cytokine involved in growth and differentiation of both B cells and eosinophils (28), after anti-CD8 treatment accompanied a reduction in joint inflammation. Further evidence for the involvement of CD8+ T cells in K/BxN mouse polyarthritis was provided by the disease relapse observed in treated mice as soon as the numbers of circulating CD8+ T cells were normalized.

Nevertheless, it was important to establish whether the permanent absence of CD8+ T cells prevented arthritis relapse. Therefore, 5-week-old K/BxN mice with established polyarthritis were thymectomized and subsequently inoculated with a high dose of CD8+ T cell–depleting mAb. Amelioration of the clinical signs of arthritis was evident after 2 weeks, and no relapses were observed in the 90-day followup period. In fact, after those 90 days, normal levels of TCR-transgenic CD4+ T cells were still present in the circulation, and the levels of B cells and plasma cells did not change when compared with those in sham-operated mice. Such observations strengthen the hypothesis that CD8+ T cells, and not only CD4+ T cells and B cells (19), are essential to the maintenance and even the initiation of chronic polyarthritis in K/BxN mice. In contrast to findings with therapies involving CD40 blockade (29), no changes were observed in the serologic levels of anti-GPI autoantibodies after any of the anti-CD8 therapies, suggesting that CD8 blockade stops/reverses ar-

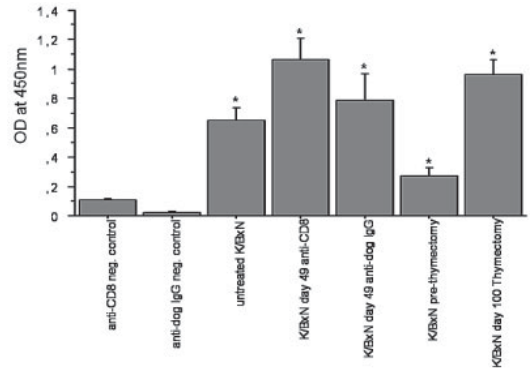

Figure 6. Blockade of CD8 does not reduce the serologic levels of anti–glucose-6-phosphate isomerase (anti-GPI) autoantibodies. Bars show the mean and SD optical density at 450 nm, measured in an anti-GPI enzyme-linked immunosorbent assay, for untreated (n = 18), anti-CD8 monoclonal antibody–treated (n = 15), or control monoclonal antibody–treated (n = 13) K/BxN mice and prethymectomized (n = 6) and postthymectomized (n = 6) K/BxN mice as well as either control monoclonal antibody–treated (n = 9) or anti-CD8 monoclonal antibody–treated (n = 6) healthy control mice. $* = P < 0.05$ versus controls. OD = optical density.

thritis progression without influencing the autoreactive B cell and plasma cell pools.

Even though the CD8+ T cells of K/BxN mice express the transgenic $V_\beta 6$ TCR, thus rendering them potentially autoreactive (as extensively described for the K/BxN mouse TCR-transgenic CD4+ T cells [19]), they have been poorly studied. A functional and phenotypic characterization of the transgenic CD8+ T cells in this mouse model is especially important in view of its larger and earlier expansion in comparison with CD4+ T cells: the CD8+ T cell pool comprised up to 85% of TCR-transgenic cells 21 days after birth and 2 weeks before arthritis was established.

CD8+ T cells are usually subdivided into particular phenotypes with characteristic effector functions, homing properties, and proliferative capacity. The expansion phase after antigen presentation is dominated by short-lived effector CD8+CD27−CD62L− T cells (T_{se}) capable of producing proinflammatory cytokines (IFNγ, TNFα, IL-2, IL-17) and cytotoxic molecules (perforin, granzyme B). These cells heavily migrate into the peripheral organs (30–32). Upon interaction with CD154 expressed on helper CD4+ T cells (33,34), subsets of effector CD8+ T cells and antigen-primed naive CD8+ T cells, respectively, develop into CD8+CD27+CD62L− effector memory cells (T_{em}) or CD8+CD27+CD62L+ central memory cells (T_{cm}) (31,35–37). While T_{em} accumulate in the peripheral organs and rapidly become effector cells upon reencounter with antigen but have poor expansion and self-renewal capacity, T_{cm} accumulate in the lymphoid organs and are capable of large expansion upon antigen reencounter and frequent self-renewal (31,37–39). Considering these functional and homing differences, it is not surprising to observe that the K/BxN mouse articular tissue showed an accumulation of the 2 effector subsets, particularly the T_{em} subset, which are more likely to participate in the local autoantigen-driven tissue destruction.

The presence of TNFα-, IL-6–, IFNγ-, IL-17–, and granzyme B–producing CD8+ T cells in the articular infiltrate and the elevated frequency of CD8+ T cells expressing the homing chemokine CXCR4 suggest that the joint-infiltrating effector CD8+ T cells might be subdivided into 2 main groups. A first group might be actively participating in joint destruction through granzyme B secretion. A second group may be involved in the recruitment and priming of other immune cells into the joint, which are the IL-17a–producing CD8+ T cells that have been described as proinflammatory but with reduced cytotoxic potential (40). Additionally, the elevated presence of both CD69-expressing CD8+ T cells, which are markers of early activation, and T_{em} in the peripheral blood of arthritic K/BxN mice suggests that there is continuous systemic activation, and eventually recruitment, of (pathogenic) CD8+ T cells in the K/BxN mouse model of spontaneous chronic polyarthritis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Souto-Carneiro had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Raposo, Pereira da Silva, Graça, Souto-Carneiro.

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Identification of Regulatory Foxp3$^+$ Invariant NKT Cells Induced by TGF- β

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Invariant NKT (iNKT) cells were shown to prevent the onset of experimental autoimmune encephalomyelitis in mice following administration of their specific TCR agonist α-galactosylceramide. We found that this protection was associated with the emergence of a Foxp3$^+$ iNKT cell population in cervical lymph nodes. We demonstrate that the differentiation of these cells is critically dependent on TGF-β in both mice and humans. Moreover, in vivo generation of Foxp3$^+$ iNKT cells was observed in the TGF-β–rich environment of the murine gut. Foxp3$^+$ iNKT cells displayed a phenotype similar to that of Foxp3$^+$ regulatory T cells, and they suppress through a contact-dependent, glucocorticoid-induced TNFR-mediated mechanism. Nevertheless, Foxp3$^+$ iNKT cells retain distinctive NKT cell characteristics, such as promyelocytic leukemia zinc finger protein expression and preferential homing to the liver following adoptive transfer, where they stably maintained Foxp3 expression. Our data thus unveil an unexpected capacity of iNKT cells to acquire regulatory functions that may contribute to the establishment of immunological tolerance. ***The Journal of Immunology*****, 2010, 185: 2157–2163.**

Natural killer T cells are innate-like lymphocytes capable of producing cytokines characteristic of Th1, Th2, or Th17 responses (1–3). They were shown to influence adaptive immunity by exacerbating or suppressing a diversity of immune disorders, such as allergy, autoimmunity, or transplantation (4–7). For instance, NKT cell activation following administration of α-galactosylceramide (α-GalCer), an NKT cell-specific agonist, can protect mice from experimental autoimmune encephalomyelitis (EAE) manifestations (5, 8).

NKT cells express a TCR and receptors typical of the NK lineage, including NK1.1 and NKG2D (7). The best-studied NKT cell subset, known as type I, classical, or invariant NKT (iNKT) cells, has a semi-invariant TCR comprising an invariant α-chain and a restricted TCR β-chain repertoire. These cells recognize glycolipids presented by CD1d (7) and develop in the thymus, where they undergo a positive selection process mediated by double positive thymocytes acting as CD1d$^+$ APCs, instead of classical MHC-expressing thymic epithelial cells (9). Thus, iNKT cells are generally considered a lineage separate from T lymphocytes, characterized molecularly by expression of promyelocytic leukemia zinc finger protein (PLZF) (10). Of note, their unique TCR remains the only distinctive feature enabling iNKT cell unambiguous detection, because they share many surface molecules with T cells, namely, CD3 and CD4 in mice, as well as CD8 in humans (7, 11).

Thus far, iNKT cell subsets identified in vivo can recapitulate in the innate immunity context some of the functions characteristic of conventional CD4 T lymphocytes, such as secretion of Th1, Th2, and Th17-type cytokines (1–3, 12, 13). However, a parallel between regulatory CD4 T cells (regulatory T [Treg] cells) and iNKT lymphocytes has not yet been described. Treg cells are characterized by expression of the transcription factor Foxp3 and potent immunosuppressive properties. They are crucial for the maintenance of an immunological self-tolerance state, modulating the activation, proliferation, and function of effector T cells, thereby preventing pathological immune responses, including allergy and autoimmunity (14). Treg cells develop in the thymus or in particular contexts at the periphery, when activated in the presence of TGF-β (15–18).

We have now identified a population of Foxp3$^+$ iNKT cells in cervical lymph nodes (CLNs) of mice protected from EAE following α-GalCer administration. We further demonstrate that murine and human Foxp3$^+$ iNKT cells can be induced in vitro following activation in the presence of TGF-β. Foxp3$^+$ iNKT cells display Treg cell phenotypic hallmarks, including CD25, glucocorticoid-induced TNFR (GITR), and CTLA-4, while retaining NKT cell characteristics, namely, PLZF expression. Moreover, Foxp3$^+$ iNKT cells can occur in vivo, following activation in the TGF-β–rich mucosal environment. Because of the strong suppressive properties displayed upon Foxp3 upregulation, we termed this new NKT cell subset "Foxp3$^+$ NKTreg cell".

Materials and Methods

Mice

C57BL/6J (H-2^b, B6), B6.Cg-*Igh^{a}Thy1^{a}Gpi1^a*/J (H-2^b, Thy1.1), BALB/cByJ (H-2^d, BALB/c), and B6.Cg-Tg(Cd4-TGFBR2)16Flv/J (H-2^b, dominant negative [dn] TGFβRII) obtained from The Jackson Laboratory (Bar Harbor, ME); B6.129S6-Rag2^{tm1Fwa}N12 (H-2^b, RAG2$^{-/-}$) obtained from Taconic Farms (Germantown, NY); and *FoxP3^{gfp}* knockin mice (H-2^b) obtained from University of Washington (Seattle, WA) were bred and maintained in specific pathogen-free conditions at the Instituto Gulbenkian de Ciência, in Oeiras, Portugal. EAE was induced with 200 μg myelin

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CLN, cervical lymph node; dn, dominant negative; EAE, experimental autoimmune encephalomyelitis; α-GalCer, α-galactosylceramide; GITR, glucocorticoid-induced TNFR; iNKT, invariant NKT; iNKTreg, invariant regulatory NKT; iTreg, induced regulatory T; MLN, mesenteric lymph node; MOG, myelin oligodendrocyte glycoprotein; nTreg, natural regulatory T; pLN, pooled lymph node; PLZF, promyelocytic leukemia zinc finger protein; Treg, regulatory T.

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oligodendrocyte glycoprotein (MOG) peptide (Biopolymers Laboratory, Harvard Medical School, Boston, MA) in 200 μg CFA (Difco/BD Diagnostics, Franklin Lakes, NJ) s.c. and 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) i.v. on days 0 and 2. Some mice received 4 μg α-GalCer (Alexis Biochemicals/Enzo Life Sciences, Plymouth Meeting, PA) on days 0 and 4. Disease severity was monitored daily and graded as follows: 1, limp tail; 2, partial hind-leg paralysis; 3, complete hind-leg paralysis; 4, front-leg weakness; and 5, moribund. In gavage experiments, 30 μg α-GalCer was delivered three times every other day.

Human subjects

Peripheral blood samples were obtained from healthy volunteers of both sexes after informed consent. The procedures were reviewed and approved by the Ethical Board of the Faculty of Medicine, University of Lisbon, Lisbon, Portugal.

Flow cytometry and cell sorting

CD1d-PBS57 tetramers were supplied by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). mAbs against mouse CD4, CD25, CD62L, CD103, CD127, CTLA-4, Foxp3, IFN-γ, IL-4, GITR, NK1.1, NKG2D, TCR-β, Thy1.1, Thy1.2; and human CD4, CD25, CD127, CD161, Foxp3, GITR, and TCR Vβ11 were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA), Beckman Coulter (Fullerton, CA), or BioLegend (San Diego, CA). For murine NKT cell enrichment, cells were incubated with unconjugated anti-CD16/32 Ab (in-house production) to block nonspecific binding to FcR and labeled with PE-conjugated CD1d-PBS57 tetramers without washing. Anti-PE magnetic beads were added, and the magnetically labeled fraction was isolated in an autoMACS Cell Separator (Miltenyi Biotec, Auburn, CA). For human NKT cell enrichment, cells were labeled with biotinylated Abs against CD14, CD19, and CD123, bound to anti-biotin magnetic beads and enriched on an autoMACS Cell Separator, the magnetically labeled fraction being discarded. Intracellular flow cytometry stainings were performed using the Foxp3 Staining Buffer Set (eBioscience) for permeabilization and fixation. Samples were analyzed on a FACSCanto I (BD Biosciences) or sorted on a FACSAria (BD Biosciences), with doublet exclusion in all experiments. Data were analyzed by FlowJo (Tree Star, Ashland, OR).

Cell cultures

Following magnetic enrichment, iNKT cells isolated from mouse spleens were sorted by FACS, and 50,000 cells per well were stimulated with 3 μg/ml plate-bound anti-CD3 (eBioscience). In some experiments, cultures were supplemented with TGF-β (5 ng/ml; R&D Systems, Minneapolis, MN), IL-2 (5 ng/ml; eBiocience), IL-15 (100 ng/ml; eBioscience), and IL-7 (5 ng/ml; R&D Systems). Human cells were stimulated with 1 μg/ml plate-bound anti-CD3 (BD Biosciences). In some conditions, cultures were supplemented with TGF-β (10 ng/ml; R&D Systems), IL-2 (20 U/ml; Roche Diagnostic Systems, Indianapolis, IN), anti–IL-12 and anti–IFN-γ (5 μg/ml; eBioscience), anti–IL-4 (5 μg/ml; R&D Systems), and anti-CD28 (2 μg/ml; eBioscience). Culture medium was RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPES, 1% penicillin/streptomycin, 1% sodium pyruvate, and 0.1% 2−ME (Invitrogen, Carlsbad, CA).

In vitro proliferation and suppression assays

Regulatory cells isolated by FACS from the spleen of naive animals (natural Treg [nTreg] cells) or from polarizing cultures (Foxp3$^+$ and Foxp3$^-$ iNKT or CD4 T cells) were cocultured in Terasaki plates (Greiner Bio-One, Frickenhausen, Germany) with mitomycin C (Sigma-Aldrich, St. Louis, MO)-treated splenocytes and responder cells (4000 naive CD4 T cells isolated from spleen; 1:1 ratio with splenocytes) stimulated with 2.5 μg/ml soluble anti-CD3 for 4 d, with addition of 1 μCi [^{3}H]thymidine (Amersham Biosciences/GE Healthcare, Sunnyvale, CA) in the last 12 h. In some experiments, 200 μg/ml anti-IL10R or 100 μg/ml anti-GITR (19) was added. In transwell assays, 100,000 naive CD4 T responder cells labeled with 5 μM CFSE (Invitrogen) were stimulated with mitomycin C-treated splenocytes (250,000) and 1 μg/ml soluble anti-CD3.

Confocal microscopy

Sorted Foxp3-GFP$^+$ cells were plated on coverslips precoated with poly-L-lysine (Sigma-Aldrich) and incubated for 1 h at 37°C. Slides were then incubated with CD1d/PBS57-PE tetramer for 1 h at 4°C, washed with ice-cold PBS, and fixed in PBS 3% paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C. Slides were mounted in DAPI Fluoromount G (Southern Biotech, Birmingham, AL) and analyzed with a laser scanning confocal microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

RNA extraction, RT, and PCR

RNA was extracted from 1,000–50,000 cells directly sorted into Buffer RLT with RNeasy Micro Kit (Qiagen, Valencia, CA), and cDNA synthesis was performed using random primers (Invitrogen) and Superscript III RT (Invitrogen). Primers (Bonsai Technologies, Orissa, India) were as follows: PLZF fwd: 5′-cagtttgcgactgagaatgc-3′, rev: 5′-ttcccacacagcagacagaa-3′; Foxp3 fwd: 5′-cccaggaaagacagcaacctt-3′, rev: 5′-ttctcacaaccaggccacttg-3′; EF1A fwd: 5′-acacgtagattccggcaagt-3′, rev: 5′-aggagccctttcccatctc-3′. PCRs were performed using the Power SYBR Green PCR Master Mix and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All PCR products were run in agarose gel.

Statistical analysis

The p values were calculated by nonparametric unpaired t test with Welch's correction.

Results

Protection from EAE upon α-GalCer treatment leads to the emergence of Foxp3$^+$ iNKT cells in CLNs

Immunization with α-GalCer had been shown to prevent the onset of EAE in wild-type mice (5, 8) (Fig. 1*A*). Because this protection is known to be iNKT cell dependent (20) (Supplemental Fig. 1), we evaluated the number and phenotype of iNKT cells at the peak of disease. Surprisingly, we found a population of Foxp3$^+$ iNKT cells in CLNs (draining the CNS) of mice protected from EAE, which was absent from other anatomical locations and in CLNs of naive animals (Fig. 1*B*, 1*C*, Supplemental Fig. 2). In contrast to Foxp3$^+$ iNKT cells, the number and frequency of Foxp3$^+$ Treg cells remained unchanged in α-GalCer–treated mice, when compared with animals with EAE or naive controls (Fig. 1*D*).

Foxp3 expression has thus far been noted to be confined to conventional αβ T cells (21–23). Consistent with this, we found no evidence for Foxp3 expression by iNKT cells from liver, spleen, pooled lymph nodes, Peyer's patches, or thymus of naive BALB/c or C57BL/6 mice (Supplemental Fig. 3). This finding strongly suggests that Foxp3 expression is not imprinted during thymic iNKT cell development. Instead, our data indicate that peripheral induction of Foxp3$^+$ iNKT cells can occur under particular pathophysiological settings, such as EAE.

TGF-β induces de novo expression of Foxp3 in iNKT cells

We investigated the capacity of iNKT cells to express Foxp3 when activated in the presence of TGF-β—a condition known to convert conventional CD4 T cells into Foxp3$^+$ "inducible" Treg cells (14, 16). Sorted iNKT and CD4$^+$CD25$^-$ T cells were stimulated with plate-bound anti-CD3 in the presence of IL-2 and TGF-β. After 3 d, Foxp3 expression was detectable in a significant proportion of both iNKT (29.35% ± 11.80) and CD4 (53.21% ± 12.03) T cell cultures (Fig. 2*A*, 2*B*). Similar results were obtained with iNKT cells from mice harboring a Foxp3-GFP knockin allele (*Foxp3^{gfp}* mice) (21) and BALB/c mice (Fig. 2*C*, Supplemental Fig. 4), and with iNKT cells sorted from the thymus, albeit the latter exhibited a lower conversion efficiency (Fig. 2*D*). Foxp3^{gfp} iNKT cells were sorted after conversion and individual cells analyzed by confocal microscopy. As shown in Fig. 2*E*, staining with CD1d tetramer loaded with the PBS57 ligand confirms that these Foxp3-expressing cells bear in their surface the invariant TCR that recognizes glycolipid Ags, a feature exclusively attributed to iNKT cells. Therefore, bona fide iNKT cells were similar to conventional CD4 T cells in their ability to upregulate the Foxp3 transcription factor when stimulated under appropriate conditions. Of note, this property was not shared by other unconventional

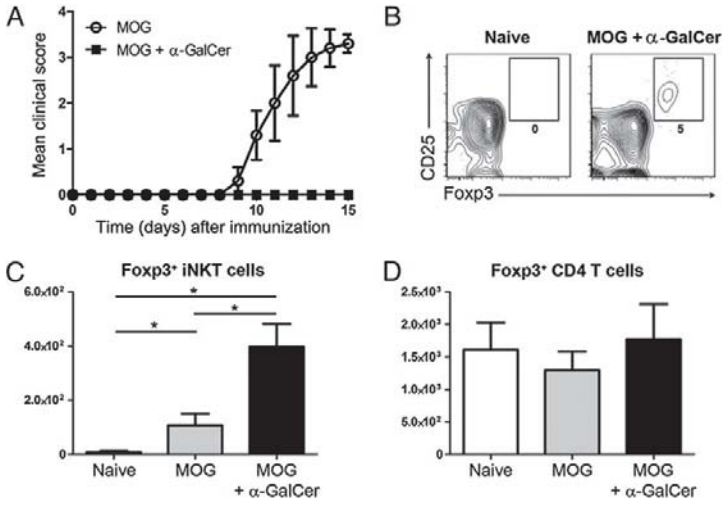

FIGURE 1. Foxp3$^+$ iNKT cells in α-GalCer–treated mice. EAE was induced in C57BL/6 mice (MOG), some of which were treated with α-GalCer (MOG + α-GalCer). *A*, Mice treated with α-GalCer were protected from EAE. $n = 5$; $p < 0.01$. *B*, Representative data from CLNs of naive and α-GalCer–treated mice (gated on iNKT cells, identified as CD1d/PBS57$^+$TCRβ$^+$ cells). *C*, Number of Foxp3$^+$ iNKT cells in CLNs, showing increased Foxp3$^+$ iNKT cell numbers in α-GalCer–treated mice. $n = 5$; $*p < 0.05$. *D*, The number of Foxp3$^+$ Treg cells in CLN remained constant. Data are representative of three independent experiments.

(non–MHC-restricted) T cells, such as γδ T cells, which failed to upregulate Foxp3 upon activation in the presence of TGF-β (M. Monteiro and J. Ribot, unpublished observations).

We analyzed the kinetics of Foxp3 induction and evaluated the stability of Foxp3 expression by iNKT cells in culture for 14 d (Supplemental Fig. 5). We observed that, despite a lower conver-

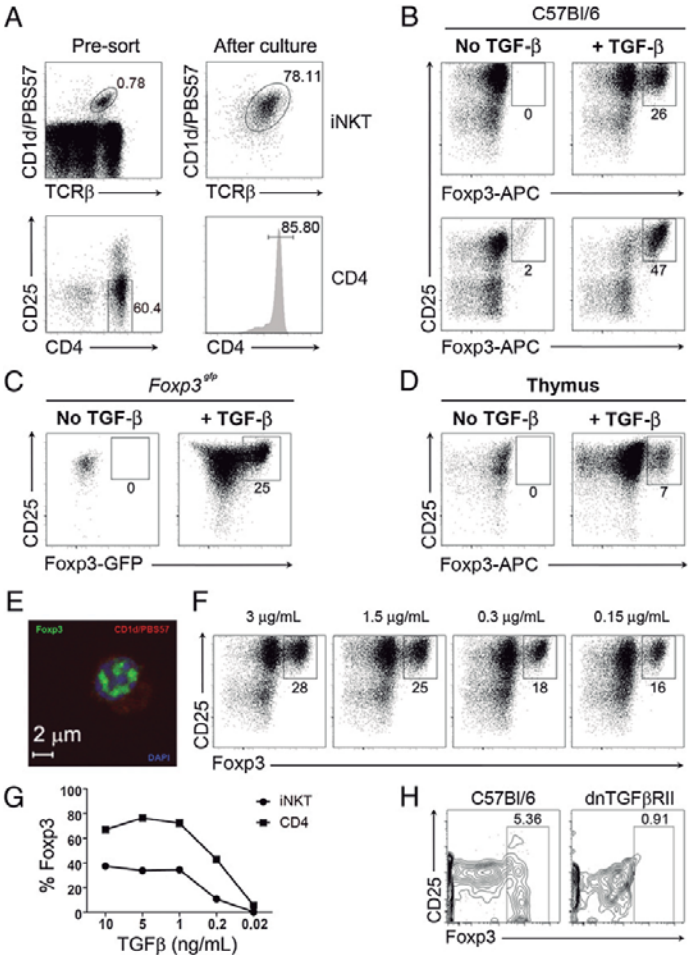

FIGURE 2. iNKT cells upregulate Foxp3 expression in the presence of TGF-β. *A–C*, Splenic iNKT cells and CD4$^+$CD25$^-$ T cells were sorted and stimulated with or without added TGF-β. *A*, Gating strategy used for cell sorting of iNKT cells (*upper panels*) and naive CD4 T cells (*lower panels*). The gating strategy used for analysis after 3 d of culture is also depicted for iNKT cells and CD4 T cells. *B*, Foxp3 expression in iNKT cells (*upper panels*) and control CD4 T cell cultures (*lower panels*) from C57BL/6 mice. *C*, Foxp3 expression in splenic iNKT cells from *Foxp3^{gfp}* knockin mice. *D*, Foxp3 expression in iNKT cells isolated from thymi of C57BL/6 mice. *E*, Foxp3 expression by iNKT cells was confirmed at single-cell level by confocal microscopy. Foxp3^{gfp}cells were FACS sorted after 3 d of culture, their invariant TCR was restained with PE-labeled CD1d/PBS57 tetramer (red), and the nucleus was counterstained with DAPI (blue). Foxp3 expression fluoresces in green (original magnification ×630). *F* and *G*, Titration of anti-CD3 or TGF-β concentration to address the impact on Foxp3 induction in splenic iNKT cells. *F*, iNKT cells cultured for 3 d in the presence of 5 ng/ml IL-2 and TGF-β, and stimulated with the indicated concentrations of anti-CD3. *G*, iNKT cells and CD4$^+$ CD25$^-$ T cells stimulated with 3 μg/ml anti-CD3 in the presence of 5 ng/ml of IL-2 and different concentrations of TGF-β after 3 d of culture. *H*, Foxp3 expression in iNKT cells from MLNs of C57BL/6 or dn TGFβRII mice following intragastric delivery of α-GalCer over 1 wk. Results are representative of three independent experiments with three mice per group.

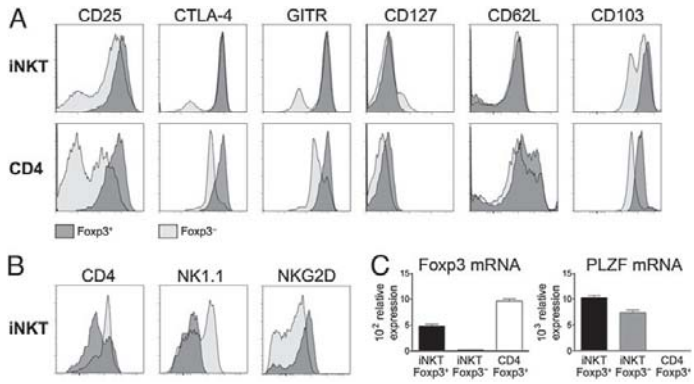

FIGURE 3. Phenotype of Foxp3$^+$ iNKT cells. *A* and *B*, iNKT cells and CD4$^+$CD25$^-$ T cells from the spleen of BALB/c (*A*) or C57BL/6 (*B*) mice were FACS sorted and cultured for 3 d in the presence of IL-2 and TGF-β. Histograms represent Foxp3$^+$ (gray) and Foxp3$^-$ (white) cells within iNKT or CD4 T cell subpopulations after conversion. Results are representative of two independent experiments. *C*, Quantification of Foxp3 and PLZF transcripts, relative to EF1A expression, from Foxp3-GFP$^+$ iNKT cells or CD4 T cells sorted after culture.

sion efficiency of iNKT cells when compared with CD4 T cell controls, iNKT cell numbers increased continuously over 14 d in culture.

We next tested the impact of TCR signal strength, costimulation, and cytokine addition on the conversion of iNKT cells into Foxp3 expressers. Maximal induction of Foxp3 was achieved with 3 μg/ml plate-bound anti-CD3 and 5 ng/ml TGF-β and IL-2, whereas further addition of IL-15 or IL-7 had little impact (Fig. 2*F*, Supplemental Fig. 4). Of note, titration of TGF-β concentration clearly revealed that this cytokine is essential for the induction of Foxp3 expression in iNKT cells (Fig. 2*G*).

TGF-β is required for expression of Foxp3 in iNKT cells in vivo

Th17 cell differentiation is critical for EAE development and requires a combination of cytokines, including TGF-β (24, 25). Accordingly, TGF-β neutralization at the time of EAE induction was shown to prevent the disease onset (26). Thus, to confirm TGF-β requirement for in vivo generation of Foxp3$^+$ iNKT cells, we adopted an alternative approach, taking advantage of the TGF-β–rich environment of the gut mucosa. We investigated whether intragastric delivery of α-GalCer could lead to the emergence of Foxp3$^+$ iNKT cells in mesenteric lymph nodes (MLNs), in the same way oral tolerance leads to de novo induction of Foxp3$^+$ Treg cells (27). We observed that α-GalCer delivery led to an accumulation of Foxp3$^+$ iNKT cells in MLNs (Fig. 2*H*). Of note, most Foxp3$^+$ iNKT cells from MLNs expressed low levels of CD25, something observed with the in vivo peripheral conversion of Treg cells in some conditions. To address whether in vivo generation of Foxp3$^+$ iNKT cells required TGF-β, we used dn TGFβRII mice (28), whose T and NKT cells are unable to transduce TGF-β signals, and observed no induction of Foxp3 expression in iNKT cells (Fig. 2*H*). Taken together, our observations suggest Foxp3$^+$ iNKT cells do not naturally arise during their development in the thymus, but can be induced in the periphery in environments where TGF-β is present.

Foxp3$^+$ iNKT cells display phenotypic characteristics of Treg cells and NKT cells

Once we established that iNKT lymphocytes could express Foxp3, we examined the phenotype of the converted cells. Many of the phenotypic characteristics of Foxp3$^+$ iNKT cells were shared with in vitro converted Foxp3$^+$ CD4 Treg cells. Both populations were predominantly CD25$^+$, CTLA-4$^+$, GITR$^+$, CD103$^+$, and IL-7Rα$^-$ (Fig. 3*A*). However, we also observed some differences between the two populations: Whereas Foxp3$^+$ CD4 T cells were heterogeneous for CD62L expression, Foxp3$^+$ iNKT cells were homogeneously CD62L^{low}. The absence of CD62L, in association with the high expression of CD103, suggests that in vivo Foxp3$^+$iNKT cells are excluded from lymph nodes and preferentially migrate to peripheral tissues. Interestingly, iNKT cells that had upregulated Foxp3 expression failed to secrete IL-4 and IFN-γ upon restimulation (Supplemental Fig. 6).

We also found that the capacity to induce Foxp3 expression was shared by CD4$^+$ and CD4$^-$ iNKT cells, as sorted CD4$^-$ and CD4$^+$ iNKT cells exhibited similar conversion efficiency (Fig. 3*B*) (M. Monteiro and C.F. Almeida, unpublished data). In addition, Foxp3$^+$ iNKT lymphocytes were NK1.1$^-$, and the majority expressed NKG2D. We also could detect the expression of PLZF, a transcription factor reported to distinguish NKT cells from conventional αβ T cells (10), in both sorted Foxp3$^+$ and Foxp3$^-$ iNKT cell subsets (Fig. 3*C*). Together, these observations indicate that induction of Foxp3 expression in iNKT lymphocytes does not corrupt their NKT cell nature.

Foxp3$^+$ iNKT cells migrate to the liver and maintain Foxp3 expression in vivo

To investigate the in vivo stability of Foxp3 expression in iNKT cells, we injected converted Foxp3-GFP$^+$ iNKT cells or control induced Foxp3-GFP$^+$ CD4 T (iTreg) cells into RAG2$^{-/-}$ mice (Fig. 4) or congenic Thy1.1 hosts (Supplemental Fig. 7). By contrast to iTreg cells, which migrated preferentially to the lymph nodes, but could also be found in the spleen and liver, 21 d after adoptive transfer iNKT cells could be detected only in the latter organ, where 50–80% (in empty hosts) or up to 10% (in wild-type congenic hosts) of iNKT cells maintained Foxp3 expression

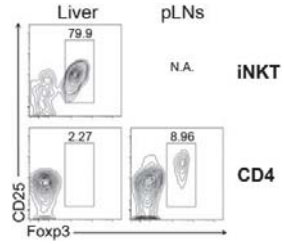

FIGURE 4. Foxp3$^+$ iNKT cells migrate to the liver. A total of 5×10^4 Foxp3-GFP$^+$ iNKT cells or CD4 T cells sorted from polarizing in vitro cultures were injected i.v. into RAG2$^{-/-}$ recipients. Data show Foxp3 and CD25 expression of iNKT cells and CD4 T cells, identified respectively as TCRβ intermediate, tetramer positive, and TCRβ$^+$CD4$^+$ cells inside the lymphocyte gate, in the liver, and in pLNs 21 d after adoptive transfer. iNKT cells were detected only in the liver. Data are representative of two independent experiments. pLNs, pooled lymph nodes.

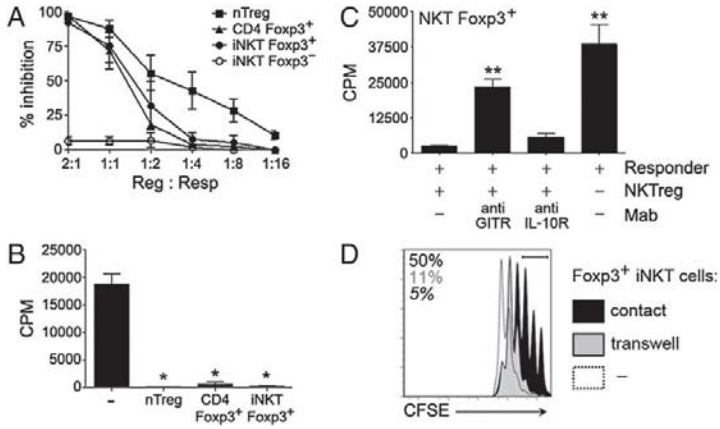

FIGURE 5. Foxp3$^+$ iNKT cells suppress T cell proliferation through a GITR-mediated contact-dependent mechanism. Foxp3$^+$ iNKT cells and Foxp3$^-$ iNKT cells sorted from the same cultures, iTreg cells (CD4 Foxp3$^+$), and nTreg cells were cocultured at different ratios with responder T cells. *A*, Average inhibition of proliferation from three independent experiments (each one with triplicates) normalized to proliferation of responder cells alone, assessed by thymidine incorporation. *B*, Representative experiment at a 2:1 ratio. $n = 3$; $*p < 0.05$. *C*, Addition of anti-GITR, but not anti–IL-10R, abrogated the suppressive effect of Foxp3$^+$ iNKT cells. $n = 4$; $**p < 0.01$. *D*, Proliferation of CFSE-labeled responder cells cultured for 72 h at 1:1 ratio with Foxp3$^+$ iNKT cells in a transwell assay, in which the two populations were cultured in contact (black histogram) or separated by a transmembrane (gray histogram). Dotted histogram shows proliferation in the absence of regulatory cells. *Upper left* numbers indicate the frequency of responder cells from the three conditions within the indicated gate.

(Fig. 4, Supplemental Fig. 7). In fact, it is becoming apparent that Foxp3 expression by conventional Treg cells is less stable than initially anticipated, especially under lymphopenic conditions (29). Our data indicate that in vivo Foxp3 stability in converted iNKT cells is not inferior to that of iTreg cells, although both populations have distinct homing potentials: Whereas CD4 Treg cells migrate predominantly to secondary lymphoid organs upon adoptive transfer, Foxp3$^+$ iNKT cells preferentially home to the liver. We thus suggest that the recruitment of regulatory iNKT cells to peripheral tissues may be important to complement and/or reinforce the local action of Treg cells (30).

Invariant regulatory NKT cells display contact-dependent GITR-mediated suppressive function

To evaluate the regulatory function of Foxp3$^+$ iNKT cells, we tested their ability to suppress proliferation of CD4$^+$CD25$^-$ "responder" cells. We used iNKT cells derived from *Foxp3^{gfp}* mice converted in the presence of TGF-β and, as controls, natural (nTreg) and iTreg cells. Titration of regulatory/responder cell ratio revealed that converted Foxp3$^+$ iNKT cells can indeed inhibit the proliferation of responder cells with similar efficiency to iTreg cells, and only slightly inferior to the efficiency of nTreg cells (Fig. 5*A*, 5*B*). Addition of anti-GITR (19), but not anti-IL-10R, neutralizing Abs to the cultures reversed suppression, indicating that GITR plays a predominant role in the regulatory function of Foxp3$^+$ iNKT lymphocytes (Fig. 5*C*). In agreement with these results, Foxp3$^+$ iNKT cells showed impaired suppressive effect compared with responder cell proliferation when cultured in a separate transwell (Fig. 5*D*), which excludes a major contribution for soluble factors to regulation. These results demonstrate that, similarly to conventional Treg cells (31, 32), induction of Foxp3 in iNKT lymphocytes endows these cells with suppressive function exerted through a contact-dependent

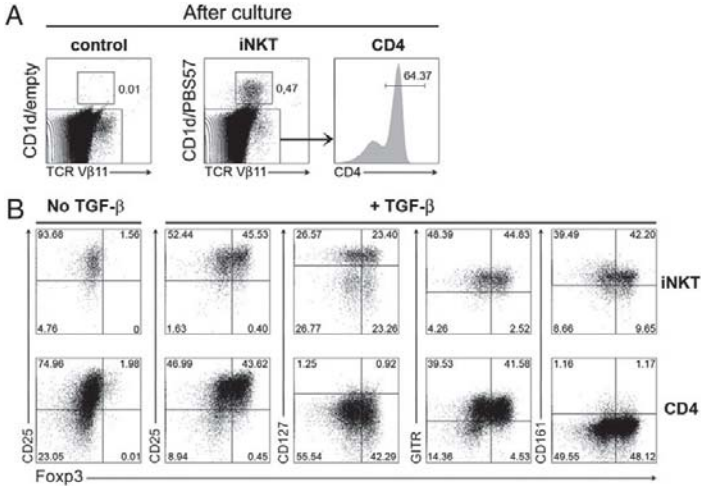

FIGURE 6. Foxp3 expression can be induced in human iNKT cells. Human iNKT cells and CD4$^+$ T cells from peripheral blood were magnetically enriched and cocultured for 5 d in the presence or absence of a conversion mixture, including or not including TGF-β. *A*, After culture, iNKT cells were identified by costaining of human CD1d/PBS57 tetramer and anti–TCR-Vβ11 Ab inside the lymphocyte gate (iNKT). Background of tetramer staining was evaluated with an empty human CD1d tetramer (control). CD4 T cells were gated inside the CD1d/PBS57-negative region (CD4). *B*, Flow cytometry data showing the coexpression of Foxp3 along with CD25, CD127, GITR, or CD161 in iNKT cell (*upper panels*) and CD4$^+$ T cell gates (*lower panels*). Results are representative of three independent experiments from different blood donors with at least three replicate cultures per condition.

mechanism mediated by GITR. This parallel with Foxp3+ Treg cells prompted us to name these Foxp3+ iNKT cells "Foxp3+ invariant regulatory NKT (iNKTreg) cells".

TGF-β–mediated induction of Foxp3 expression in human iNKT cells

Finally, we also evaluated whether Foxp3 expression could be induced in human iNKT cells. Given the lower frequency of iNKT lymphocytes in human peripheral blood (31), we enriched total T cells by magnetic separation and cultured these bulk populations in polarizing conditions that included TGF-β and a mixture of neutralizing Abs against IL-12, IFN-γ, and IL-4 (32) (Fig. 6*A*). After 5 d of culture, up to 40% of human iNKT cells had upregulated Foxp3, an efficiency of conversion comparable to that of conventional CD4+ T cells (Fig. 6*B*). The converted human Foxp3+ NKT cells were CD25+, GITR+, and predominantly CD161+, whereas CD127 was expressed by approximately half of the Foxp3+ iNKT cells. Critically, these data establish that the induction of Foxp3 in NKT cells is a conserved phenomenon between rodents and humans.

Discussion

Among hematopoietic cells, Foxp3 expression has been claimed to be restricted to conventional T cells (24–27, 33). We now describe, for the first time, that iNKT and CD4 T lymphocyte populations share a similar competence in responding to a tolerogenic cytokine environment, namely, TGF-β, by expressing Foxp3 and undergoing functional specialization toward a regulatory phenotype. It should be noted, however, that Foxp3 induction upon activation in the presence of TGF-β is not a common feature of all lymphocytes: In particular, we were unable to convert sorted γδ T cells by CD3 stimulation in the presence of TGF-β (M. Monteiro and J. Ribot, unpublished observations). In addition, this potential appears to be a conserved feature of iNKT cells, as we were able to induce Foxp3 expression in samples from different murine strains and from human beings.

Our results clearly demonstrate that TGF-β is the critical cytokine promoting Foxp3 upregulation in iNKT cells, as described for CD4+ T cell conversion into Foxp3+ Treg cells (15–18). As a consequence, we took advantage of the TGF-β–rich environment of the gut to assess in vivo conversion of Foxp3+ iNKT cells. In fact, it has already been described that oral delivery of an Ag can lead to a state of mucosal tolerance in which Ag-specific T cells are locally converted into Treg cells (27). In this paper, we show that intragastric delivery of α-GalCer induces the emergence of Foxp3+ iNKTreg cells in MLNs. However, this process was impaired in mice with NKT cells unable to integrate the signals delivered through the TGF-βR, thus establishing a crucial role for TGF-β in the in vivo conversion of Foxp3+ NKT cells. Therefore, although oral tolerance to proteic Ags, such as OVA, can be achieved in the absence of iNKT cells, as it has been shown using TCR-transgenic RAG-deficient mice (27), our results suggest that iNKT cells in the gut, exposed to glycolipid ligands, can convert into Foxp3+ cells and synergize with local Treg cells in the promotion of tolerance to food Ags.

Most reports have shown that Foxp3+ Treg cells suppress immune responses through cell contact-dependent mechanisms (34, 35). Several surface molecules preferentially expressed by Treg cells, such as GITR, have been implicated in their suppressive function (31, 32). We found that NKTreg cells can also suppress naive T cell proliferation by a GITR-dependent, IL-10–independent mechanism. Moreover, iNKTreg cells were unable to suppress responder cell proliferation when physically separated in a transwell, which excludes the additional contribution of soluble regulatory factors.

Although iNKT cells expressing Foxp3 have never been found in naive mice (21), the presence of this population has never been investigated in the context of an immune response. For instance, iNKT cells were reported to play important regulatory roles in the development of anterior chamber-associated immune deviation, transplantation tolerance, and prevention of autoimmunity, such as EAE, as lack of iNKT cells or decreased iNKT cell numbers compromise tolerance. However, in none of these studies was Foxp3 expression assessed in iNKT cells (33). Importantly, iNKT cell surface molecules largely overlap with those expressed by conventional CD4 T lymphocytes, and Foxp3+ iNKT cells downregulate expression of NK1.1, a molecule often used in previous studies to distinguish NKT cells from T lymphocytes. Therefore, it is reasonable to assume that without an unambiguous identification with a specific tetramer, Foxp3+ iNKT cells could have been erroneously detected as conventional Treg cells. Furthermore, until the development of a conditional knockout mouse lacking Foxp3 exclusively in iNKT cells—for instance, based on a PLZF-driven Cre expression system—it will not be possible to discriminate between the contribution of NKTreg cells and that of Treg cells in immune pathology. Although this topic surely deserves further investigation, the results presented in this paper clearly establish a new type of iNKT cell characterized by Foxp3 expression and immune regulatory properties similar to those of conventional Treg cells, one that might contribute to the maintenance of immune tolerance in the periphery.

Finally, the strong immunosuppressive properties displayed by NKTreg cells raise the possibility of their therapeutic application in the control of immune-mediated diseases, in particular, liver immune-mediated inflammation. Given their invariant specificity, Foxp3+ NKTreg cells are assumed to have a general immunosuppressive action. It should be noted, however, that polyclonal Treg cell populations are likely to have, at least in part, a similar nonspecific immunosuppressive effect, possibly due to cross-reactivity with self-Ags (36). Indeed, these nonspecific effects can serve as the basis of Treg cell suppression in lymphopenia-driven proliferation and graft-versus-host disease (36–38). Notably, the invariant TCR expressed by Foxp3+ iNKT cells might provide an advantageous, universally applicable method for their isolation, conversion, and expansion, regardless of genetic background or pathology.

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Disclosures

The authors have no financial conflicts of interest.

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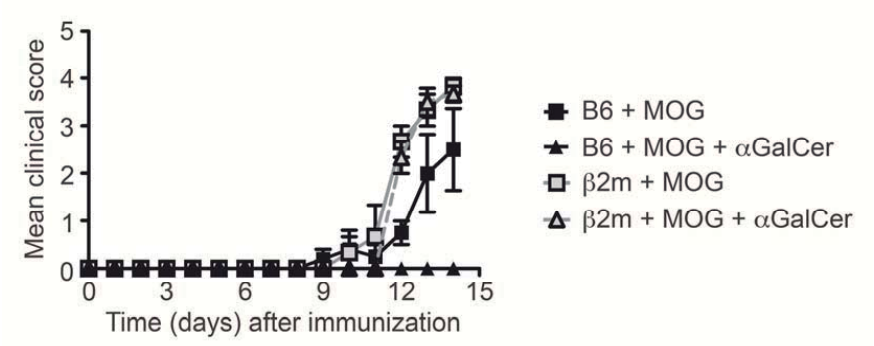
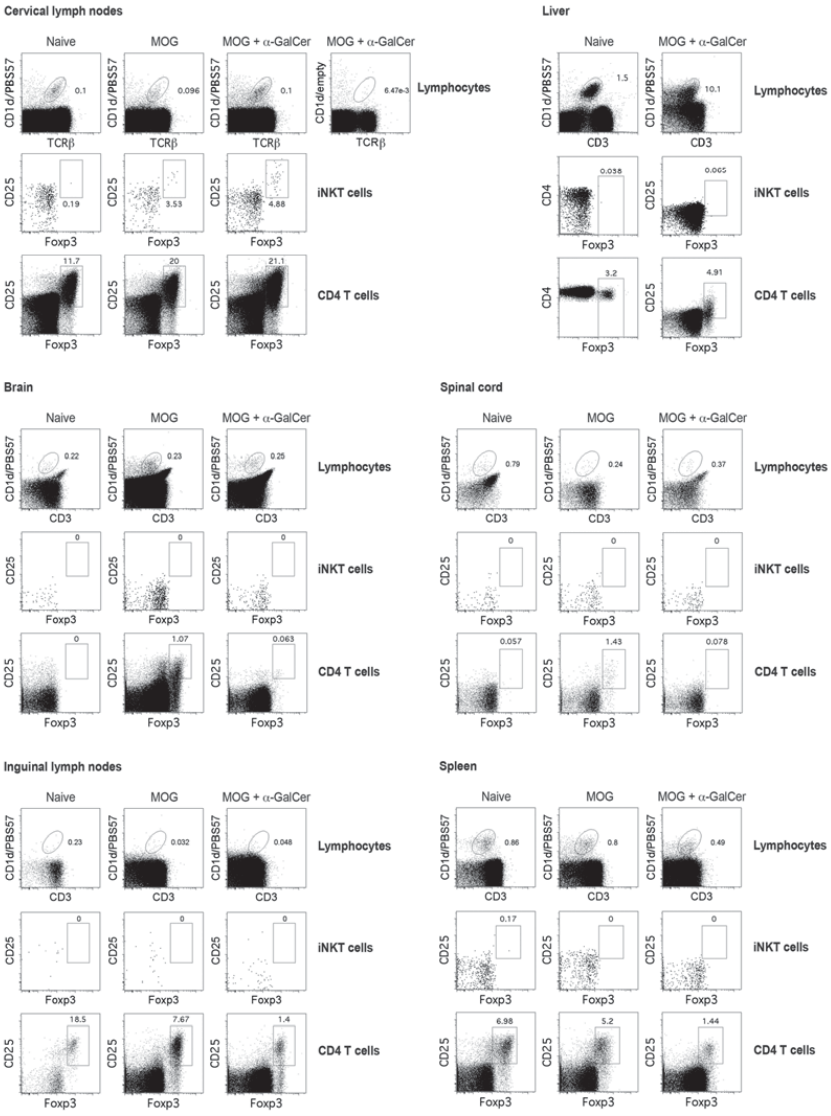
Supplemental Figure 1



α-GalCer administration does not prevent EAE manifestations in mice lacking iNKT cells. EAE was induced in C57Bl/6 (B6+MOG) and in β2-microglobulin deficient mice (β2m+MOG) mice, some of which were treated with α-GalCer. Administration of α-GalCer prevented the development of EAE in B6 but not in β2-microglobulin deficient mice, which lack iNKT cells (n=5, $P<0.01$).

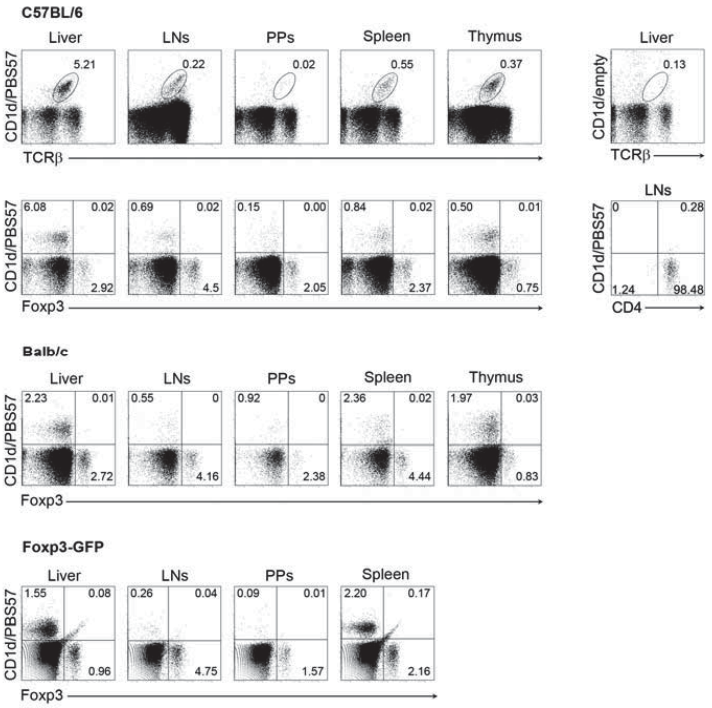
Supplemental Figure 2



Foxp3+ iNKT cells generated during EAE are only detectable in cervical LNs. EAE was induced in C57Bl/6 mice (MOG), some of which were treated with α-GalCer (MOG+α-GalCer). Panels show representative flow cytometry data of cells isolated

from different organs 14 days after immunization with MOG, showing iNKT identification with the specific tetramer and anti-CD3 or TCRβ, and Foxp3 staining within iNKT and CD4 T cell gates. A control dot plot obtained with an empty CD1d tetramer is also represented. Data are representative of three independent experiments.

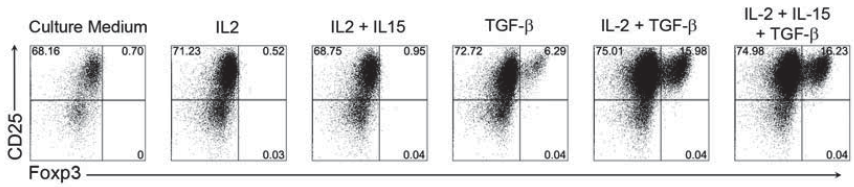
Supplemental Figure 3



iNKT cells from naïve C57Bl/6, Balb/c, and Foxp3^{gfp} knockin mice lack Foxp3 expression. Analysis of Foxp3 expression in lymphocytes isolated from the liver, pooled LNs, Peyer's Patches (PP), spleen and thymus of C57Bl/6, Balb/c mice and Foxp3^{gfp} naïve mice. iNKT cells were identified by co-staining with CD1d/PBS57 tetramer and a pan TCR-β antibody inside the lymphocyte gate (upper row). Background staining of iNKT cells was assessed for every organ using an empty CD1d tetramer (shown for liver only). Foxp3 expression was analyzed through intracellular antibody staining or based on GFP fluorescence (in Foxp3^{gfp} mice). Foxp3^{+} cells were only present among the CD1d/PBS57^{-} cells. We confirmed these Foxp3^{+} cells as being

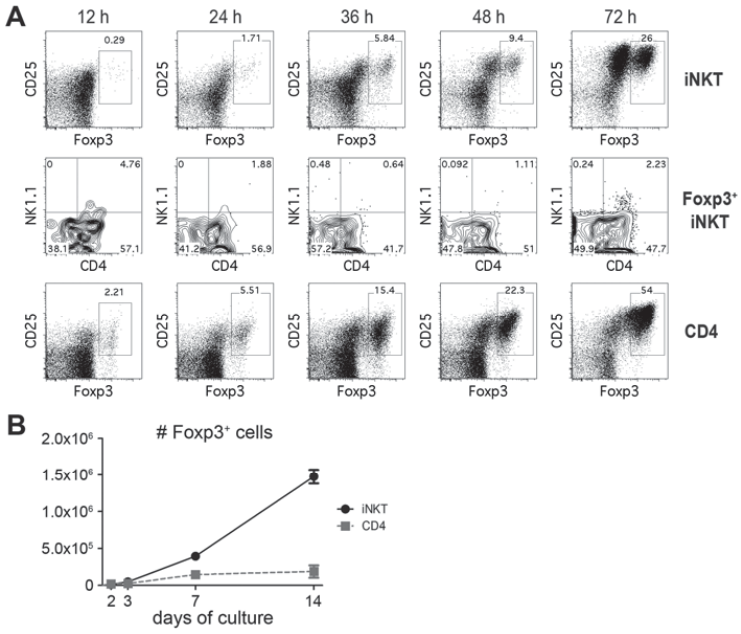
$CD4^+$ T lymphocytes (right dotplot, shown only for LNs). Results are representative of three mice from two independent experiments.

Supplemental Figure 4



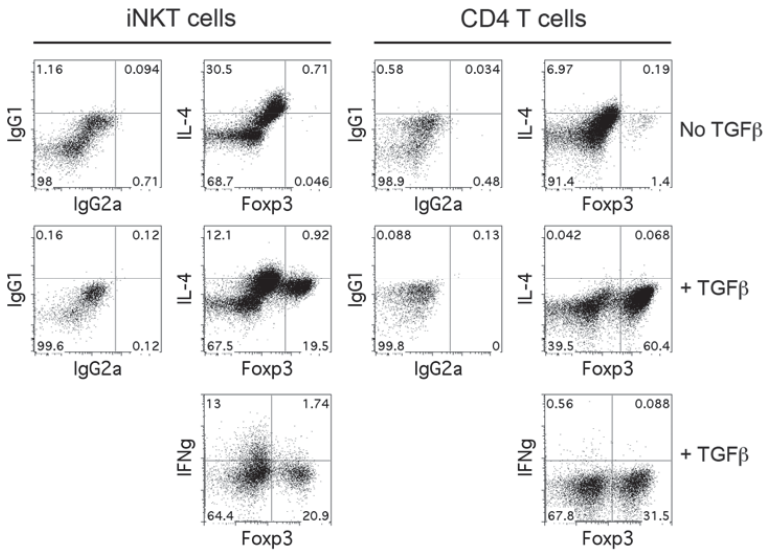
Culture of Balb/c iNKT cells under different cytokine conditions. iNKT cells isolated from the spleen of Balb/c mice were FACS-sorted according to the co-expression of a CD1d/PBS57 tetramer and a pan TCR-β antibody, and stimulated with 3 μg/mL of plate-bound anti-CD3 for 3 days in the indicated conditions. Results are representative of three independent experiments.

Supplemental Figure 5



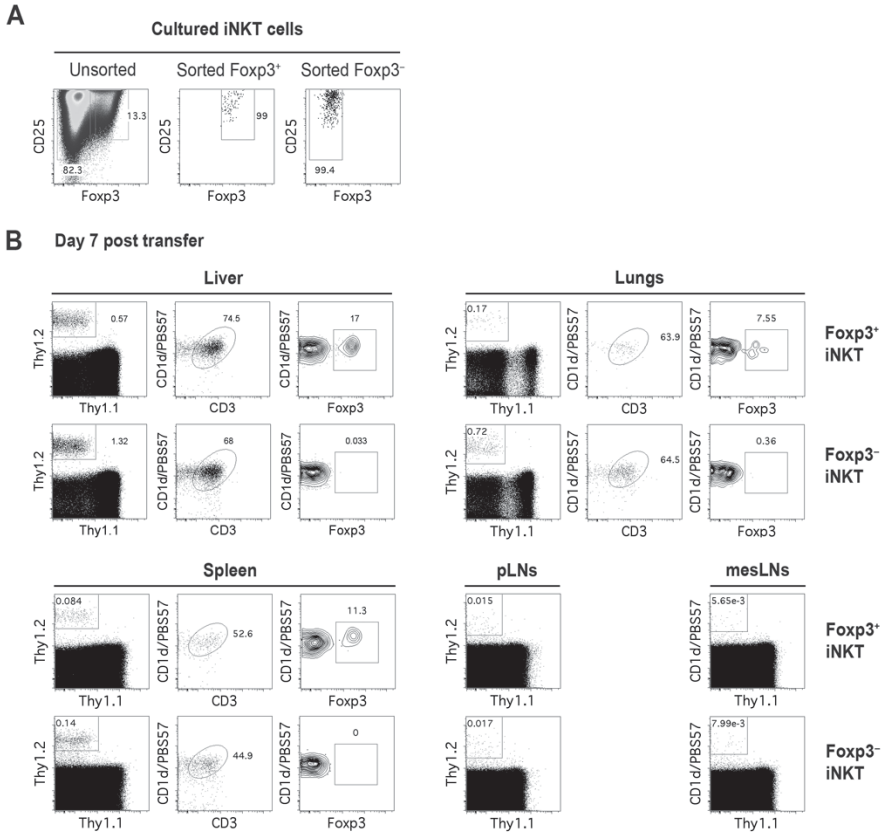
Time-course analysis of Foxp3 induction and stability in iNKT cells. iNKT and naïve CD4 T cells isolated form the spleen of naïve C57BL/6 mice were sorted, stimulated *in vitro* in presence of IL-2 and TGF-β and Foxp3 expression analyzed at different time points. *A* Kinetics of Foxp3 upregulation at the indicated time-points in the first 72 hours of culture. The profile of NK1.1 and CD4 expression in Foxp3$^+$ iNKT cells is shown in panel *A*, middle row. *B,* Graphs represent kinetics of the number of Foxp3$^+$ iNKT or CD4 T cells cultured for 14 days in presence of TGF-β. Culture medium was replaced every 3 days and wells split when cells reached confluence. Data is representative of at least 2 independent experiments performed with triplicates.

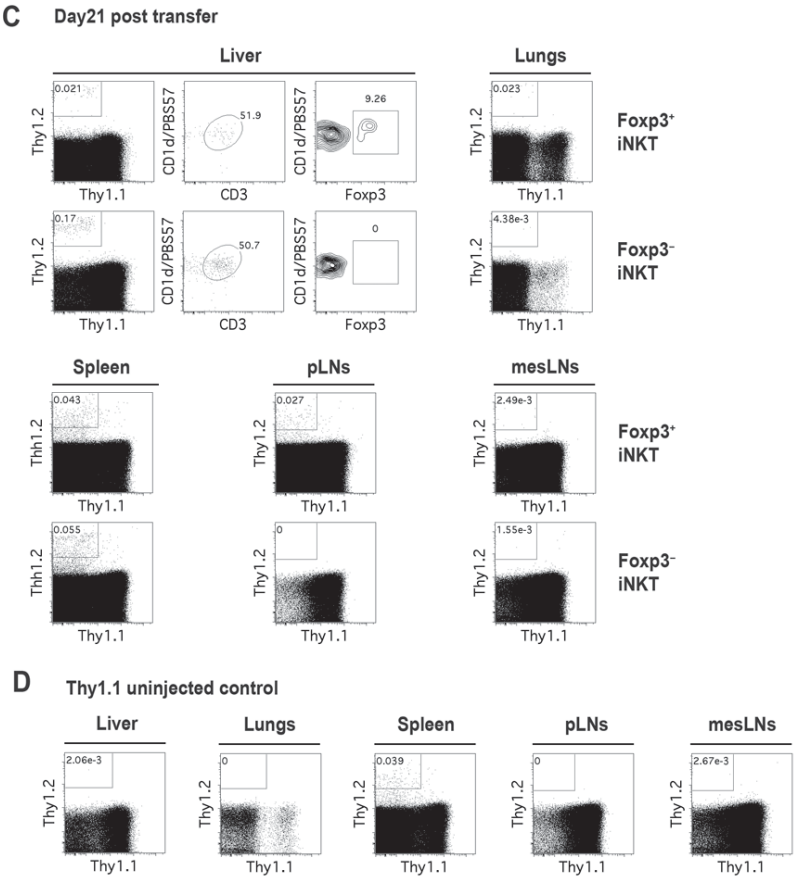
Supplemental Figure 6



Foxp3$^+$ iNKT cells fail to produce IL-4 and IFN-γ. After 3 days of *in vitro* stimulation in presence of IL-2 and TGF-β, iNKT cells were stimulated with PMA/ionomycin and IL-4, IFN-γ and Foxp3 were detected by flow cytometry. The figure shows the co-staining of cytokines and Foxp3, or the respective isotype controls, in both iNKT (left dot plots) and CD4 T cells (right dot plots) cultured in absence (top row) or presence of exogenous TGF-β (second and third rows). Data are representative of two independent experiments performed with triplicate cultures per condition.

Supplemental Figure 7





***In vivo* distribution of adoptively transferred Foxp3$^+$ iNKT cells.** 1×10^5 Foxp3-GFP$^+$ or Foxp3-GFP$^-$ iNKT cells (sorted from the same 5-day cultures) were transferred intravenously into congenic Thy1.1 naïve hosts. At day 7 or 21 after adoptive transfer, mice were sacrificed and cells recovered from different tissues were analyzed by flow cytometry. *A*, Dot plots from the cell-sorting of iNKT cultures, in which Foxp3$^-$ and Foxp3$^+$ were isolated based on GFP fluorescence. Purity of sortings is also shown. *B,C*, Representative FACS data of cells isolated from the indicated organs at day 7 (B) or 21 (C) post-transfer. To identify donor cells, we first excluded background and host cells

through MHC class II and Thy1.1 staining, respectively, inside the lymphocyte gate, gating afterwards on Thy1.2$^+$ cells and then in tetramer and CD3 co-expressers. Foxp3 expression was translated by GFP signal. *D*, Dot plots show the co-staining of Thy1.1 and Thy1.2 of cells isolated from the indicated organs of unmanipulated Thy1.1 control mice gated as in B and C.

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Modulation of IL-17 and Foxp3 Expression in the Prevention of Autoimmune Arthritis in Mice

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Abstract

Background: Rheumatoid Arthritis (RA) is a chronic immune mediated disease associated with deregulation of many cell types. It has been reported that different T cell subsets have opposite effects in disease pathogenesis, in particular Th17 and Treg cells.

Methodology and Findings: We investigated whether non-depleting anti-CD4 monoclonal antibodies, which have been reported as pro-tolerogenic, can lead to protection from chronic autoimmune arthritis in SKG mice – a recently described animal model of RA – by influencing the Th17/Treg balance. We found that non-depleting anti-CD4 prevented the onset of chronic autoimmune arthritis in SKG mice. Moreover, treated mice were protected from the induction of arthritis up to 60 days following anti-CD4 treatment, while remaining able to mount CD4-dependent immune responses to unrelated antigens. The antibody treatment also prevented disease progression in arthritic mice, although without leading to remission. Protection from arthritis was associated with an increased ratio of Foxp3, and decreased IL-17 producing T cells in the synovia. *In vitro* assays under Th17-polarizing conditions showed CD4-blockade prevents Th17 polarization, while favoring Foxp3 induction.

Conclusions: Non-depleting anti-CD4 can therefore induce long-term protection from chronic autoimmune arthritis in SKG mice through reciprocal changes in the frequency of Treg and Th17 cells in peripheral tissues, thus shifting the balance towards immune tolerance.

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

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Introduction

Rheumatoid arthritis (RA) is a common chronic autoimmune inflammatory disease characterized by destruction of the synovial joints, leading to progressive disability, increased co-morbidity and premature mortality [1,2]. Both genetic and environmental factors are known to contribute to the development of the disease [3]. RA is characterized by a complex immune mediated response with the participation of many cell types including $CD4^+$ T cells [4,5,6], such as the IL-17 producing Th17 subset, which have been shown to play an important role in the pathogenesis of the disease [7,8,9]. The participation of $CD4^+$ T cells in the pathogenesis of RA, namely by influencing other key cellular mediators of the disease (such as B cells or macrophages), has prompted the development of therapeutic strategies targeting this lymphocyte population [10,11,12,13]. Monoclonal antibodies (MAbs) targeting key T cell molecules (such as co-receptor and co-stimulation) have been suggested as drugs capable of achieving long-term protection from the disease, with the potential of leading to immune tolerance, following a short treatment [14]. Indeed long-term transplantation tolerance can be induced in mice following CD4 or co-stimulation blockade [15,16,17].

The most commonly used mouse models for autoimmune arthritis – such as collagen-induced arthritis – have been instrumental in the development of new therapies, such as the blockade of key cytokines, such as TNF. However, arthritis in these mice is self-limited and, as such, pre-clinical studies of putative tolerogenic regimens aiming for long-term effects have been hampered by the lack of suitable animal models of chronic autoimmune arthritis that are not TCR transgenic.

SKG mice, harboring a mutation in ZAP-70 rendering T cells more resistant to activation and thus interfering with appropriate negative selection in the thymus, have been recently described as developing chronic autoimmune arthritis with several characteristics resembling RA [18]. Arthritis in SKG mice has a centripetal course starting with small finger joints, eventually leading to histological changes and bone destruction similar to RA [19]. The incidence and severity of the disease is greater in females, with most mice developing rheumatoid factor (RF), and some animals displaying extra-articular lesions similar to rheumatoid nodules and pneumonitis [18]. Although $CD4^+$ T cells, and its Th17 subset, are important in the pathogenesis of arthritis in SKG mice, other cell populations, such as B cells, participate in the disease as suggested by the production of RF in these animals [18].

Our data reports the long-term protection from chronic autoimmune arthritis following a short course of non-depleting anti-CD4 MAb in SKG mice, associated with decreased IL-17 and increased Foxp3 expression in the synovial tissue. Furthermore, the non-depleting nature of the therapeutic MAb preserves the immune competence of treated mice.

Methods

Ethics Statement

All experiments involving animals were approved by the Animal User and Ethical Committees at the Instituto Gulbenkian de Ciencia, according with directives from Direccao Geral Veterinaria (PORT 1005/92). Mice were bred and maintained under specific pathogen free (SPF) conditions.

Mice

BALB/c, DO11.10.RAG1$^{-/-}$, and SKG mice (generously provided by Professor Shimon Sakaguchi, Kyoto, Japan). Experimental animals were between 8–10 weeks of age and sex matched.

Autoimmune arthritis induction and anti-CD4 treatment

BALB/c and SKG mice were injected intraperitoneally (i.p.) with a single shot of 3mg curdlan per mouse. Treated mice were injected with 1 mg anti-CD4 on days 0 (the day of curdlan injection), 2 and 4. Mice treated at disease onset, were injected with 3 shots of 1 mg anti-CD4 every other day, from the day they have reached a clinical score of 0.5.

Clinical assessment of arthritis

Joint swelling was monitored in blinded cages by two independent observers and scored as described elsewhere [18]: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist, ankle, or base of tail; and 1.0, severe swelling of wrist, ankle or base of tail. Scores for all joints were totaled for each mouse (with a maximum score of 5 corresponding to severe swelling of the four paws and base of tail).

Antibodies and reagents

Curdlan (Wako, Japan) and Zymosan-A (Sigma-Aldrich, USA) were dissolved in sterile PBS at 15 mg/ml and 20 mg/ml, respectively. Non-depleting anti-CD4 (YTS177) and the isotype control anti-dog CD4 (YKIX302) MAbs were produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland), purified by 50% ammonium sulfate precipitation, dialyzed against PBS, and purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Cytokine analysis

Evaluation of serum cytokines was performed using the mouse inflammation cytometric bead array (BD Biosciences, San Diego, CA), with beads specific for IL-6, TNF, IFN-γ, monocyte chemoattractant protein-1 (MCP-1), and IL-10. IL-17 was quantified by ELISA using a kit from R&D Systems. Cytokine concentrations were measured in duplicates, and compared with standard curves, according to manufacturer instructions.

Quantification of rheumatoid factors

Serum levels of rheumatoid factor were measured by ELISA as previously described [18], using 5 μg/ml mouse IgG to coat the plates, and 1 μg/ml anti-mouse-IgM-HRP for detection (Southern Biotech, Birmingham, USA).

Determination of ovalbumin-specific immune responses

On day 30 following anti-CD4 treatment, or treatment with an isotype control, the mice were immunized with two injections two weeks apart, of 20 μg ovalbumin (OVA, grade V; Sigma, St Louis, USA), in 2.0 mg of endotoxin-free aluminum hydroxide (alum, Alu-gel-S, Serva, Heidelberg, Germany), and sacrificed one week after the last immunization. Non-immunized (naïve) mice were maintained as negative controls. The serum concentration of OVA-specific immunoglobulins was determined by ELISA using an OVA-specific IgG1 kit (SouthernBiotech, Birmingham, USA) with anti-OVA IgG1 standard from Serotec (Oxford, UK).

Histology

Ankle and metatarso-phalangical (MTP) joints were collected and cryopreserved in OCT (Sakura, NL). Cryosections were stained with hematoxilin-eosin according to standard procedures.

Flow cytometry

Cells were stained for flow cytometric analysis with the following fluorochrome-labeled monoclonal antibodies: CD3 (145-2C11), CD4 (RM4-5), CD25 (PC61.5), and Foxp3 (FJK-16s) from eBiosciences or BD Biosciences. Intracellular cytokines were investigated in lymphocytes activated for three hours in PMA-ionomycin in the presence of Brefeldin-A. Monoclonal antibodies specific for IFN-γ (XMG1.2), IL-17A (eBio17B7), and IL-10 (JES5-16E3) were used (all from eBiosciences).

Th17-polarization assays

OVA-specific CD4^{+} T cells from DO11.10.RAG1$^{-/-}$ mice were purified by magnetic separation with CD4 (L3T4) microbeads (Miltenyi Biotec, Germany). Cell purities were between 92–96%. The T cells were cultured for 5 days with bone marrow derived DCs, 0.1 μM OVA peptide (New England Peptide LLC, USA), and 10 μg/ml anti-CD4 (YTS177) in IMDM 5% FBS (Invitrogen), 1% Pen/Strep (GibCo), 0.1% β-Mercaptoethanol (GibCo), 1 ng/ml TGF-β (R&D systems), 20 ng/ml IL-6 (R&D systems), 10 ng/ml IL-1β (Ebiosciences), and 10 μg/ml anti-INFγ (R46A2). At the end of the culture the cells were harvested, and processed for flow cytometry.

RNA extraction and real time PCR

Total RNA was extracted from synovial tissue, dissected from ankle joints, using lysis buffer, and following the tissue RNA kit instructions (Omega bio-tek, USA). Foxp3 and IL-17 were quantified by real time PCR, performed on the ABI Prism® 7000 sequence detection system (Applied Biosystems, USA). The relative mRNA levels of the target genes were normalized against CD3. CD3, Foxp3 and IL-17 primers are described elsewhere [20,21,22].

Statistics

Statistical significance was determined using the two-tailed non-parametric Student's t test (Mann-Whitney U). P values <0.05 were deemed significant.

Results

SKG mice develop chronic autoimmune arthritis upon systemic curdlan immunization

Although initial reports have suggested that SKG mice develop chronic autoimmune arthritis spontaneously [18], it was later confirmed that disease induction requires exposure to yeast wall extract (zymosan) or purified β-glucans, like curdlan or laminarin, acting through the pattern recognition receptor Dectin-1 [23].

We confirmed that a single intraperitoneal injection of 2 mg Zymosan (not shown) or 3 mg Curdlan is sufficient to induce chronic disease in SKG mice, but not in wild-type controls (Figure 1A). Both male and female mice developed arthritis, although the disease was more severe in females (Figure 1B). Based on these data we decided to use curdlan in female SKG mice in subsequent experiments.

Non-depleting anti-CD4 treatment prevents the onset of autoimmune arthritis

To assess whether non-depleting anti-CD4 MAbs, suggested in previous studies as having tolerogenic potential, can lead to long-term beneficial effects in chronic autoimmune arthritis, female SKG mice were treated with non-depleting anti-CD4 together with curdlan. Anti-CD4 treatment was effective in preventing the development of autoimmune arthritis ($n = 5$, $P<0.001$, Figure 2A). Since arthritic SKG mice are known to produce high titres of RF (IgM anti-IgG), we quantified serum RF. We found that serum RF were below the limit of detection in all mice treated with anti-CD4 (Figure 2B).

Furthermore, animals treated with anti-CD4 not only remained without clinical manifestations of the disease, but their joints were free from inflammatory cell infiltrates. Indeed, histological sections of ankle and MTP joints from anti-CD4 treated mice showed normal joint tissues, without inflammatory infiltration or bone erosions, compared to the curdlan induced arthritic mice (Figure 2C).

We also evaluated the presence of pro-inflammatory cytokines in arthritic mice compared to anti-CD4 treated animals. We observed a significant decrease in the concentration of IL-6, TNF, MCP-1 and IFN-γ in sera from anti-CD4 treated mice, suggesting that a treatment targeting T cells can have an impact on additional cell types producing those cytokines (Figure 2D). It should be noted that IL-6, which has been associated with Th17 differentiation, is known to be critical for the pathogenesis of chronic autoimmune arthritis in SKG mice [24], and was not detectable in any of the anti-CD4 treated animals. Despite the reported association of IL-17 with arthritis in SKG mice, this cytokine was not detected in sera from arthritic animals, remaining below detection level in all groups (Figure 2E). This observation is in accordance with the proposed local effect of this inflammatory mediator.

Previous studies have suggested a protective role for IL-10 in this murine model of autoimmune arthritis [25]. However, we did not find any significant difference in the serum levels of IL-10, between arthritic and anti-CD4 treated mice ($n = 10$, $P>0.05$, Figure 2E).

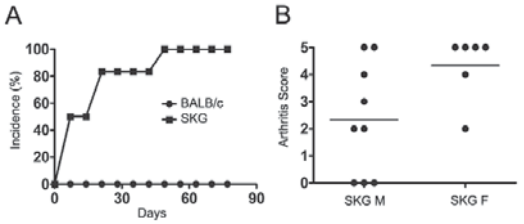

Figure 1. SKG mice develop chronic autoimmune arthritis upon induction with curdlan. (A) Arthritis incidence in female SKG and BALB/c mice after a single i.p. injection of 3 mg curdlan ($n = 6$). Curdlan was able to induce chronic arthritis in SKG but not in BALB/c mice. Each point represents the median per group. (B) Clinical score of 5 month-old male (M) and female (F) SKG mice 90 days following disease induction with curdlan. Data are from two independent experiments.
doi:10.1371/journal.pone.0010558.g001

Non-depleting anti-CD4 alters the balance of Treg/Th17 cells in the synovial tissue and draining LNs

It has been reported that the anti-CD4 MAb used in our study (YTS177) has a non-depleting isotype [26]. We confirmed the non-depleting nature of the MAb as neither the splenic frequency (Figure 3A and B) nor the total number (not shown) of $CD4^+$ T cells were reduced in animals treated with anti-CD4.

Transplantation tolerance achieved with non-depleting anti-CD4 has been associated with the induction of Treg cells, both in the spleen and within tolerated transplants [27,28,29]. We investigated if changes in Treg frequency could be seen in the spleen of anti-CD4 treated mice. We found that there was no change in T cell subpopulations of anti-CD4-treated animals, namely $Foxp3^+$ Treg cells, with a similar frequency of these cells in mice exposed to curdlan in the presence or absence of anti-CD4 treatment (Figure 3A and B). In addition, the frequency of IL-17, IFN-γ or IL-10-producing T cells, identified by intracellular cytokine staining, remained constant in all groups of animals, and below 2% of the $CD4^+$ T cells (not shown).

As our data show that anti-CD4 treatment can prevent joint inflammation, even though T cell subpopulations in secondary lymphoid organs appear to be unaffected, we investigated whether anti-CD4 treatment was leading to alterations in the T cell subpopulations within the synovial tissue of protected animals. Given the technical limitations for the direct enumeration of individual T cells from mouse synovial tissue, we used the expression of Foxp3 and IL-17 mRNA as surrogate markers for the relative frequency of, respectively, Tregs and Th17 cells. For this purpose Foxp3 and IL-17 mRNA expression was measured by quantitative real time PCR and normalized to CD3 expression (thus controlling for different numbers of infiltrating T cells in different samples, as the arthritic synovium contains greater numbers of T cells). This method, of using CD3 expression for normalization of different numbers of infiltrating T cells in tissues with few lymphocytes has been established for other tissues with small numbers of T cells, such as skin grafts in mice [22]. We observed that while IL-17 expression among synovial T cells (i.e. IL-17/CD3 mRNA ratio) was reduced in mice treated with anti-CD4 and consequently protected from inflammatory manifestations of the disease, the expression of Foxp3 among synovial T cells was increased in the same conditions ($n = 4$, $P<0.05$, Figure 3C). Thus, the Foxp3/Th17 ratio in the synovial tissue is substantially shifted following anti-CD4 treatment. In addition, the analysis of popliteal LNs, draining affected joints, showed a reduction in the frequency of $IL\text{-}17^+$ T cells in anti-CD4-treated mice ($n = 8$, $P<0.05$) while the frequency of $Foxp3^+$ cells remained similar in both groups of animals (Figure 3D).

CD4-blockade prevents *in-vitro* Th17 polarization while favoring Foxp3 expression

Although non-depleting anti-CD4 has been widely studied for the induction of transplantation tolerance its exact mechanism of action has not been fully characterized. In experiments performed with TCR-transgenic mice devoid of $Foxp3^+$ Treg cells, it was previously shown that CD4-blockade could directly lead to peripheral conversion of T cells towards $Foxp3^+$ Treg cells thus achieving transplantation tolerance [22]. However, it was never assessed whether CD4-blockade could directly interfere with Th17 polarization, or whether a reduction in Th17 cells (as we observed *in vivo*) would be secondary to Treg-mediated suppression.

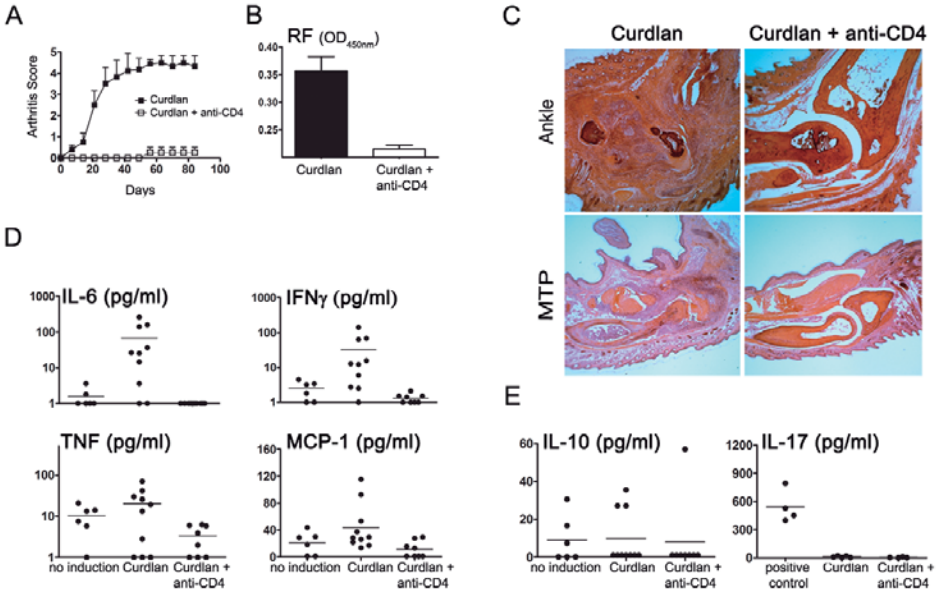

Figure 2. Non-depleting anti-CD4 MAb prevents the onset of autoimmune arthritis. (A) Female SKG mice were immunized with 3mg curdlan i.p. together with 1 mg non-depleting anti-CD4 or an isotype control. The MAb was administered again on days 2 and 4. Anti-CD4 treated mice were protected from the development of autoimmune arthritis (n = 6, $P<0.001$). Data, represented as mean ± SEM, are from two independent experiments. (B) Serum concentration of rheumatoid factor (RF) was measured by ELISA. Mice treated with anti-CD4 showed significantly lower levels of RF (n = 6, $P<0.001$) (C) Histological sections stained with eosin-hematoxilin from the ankle and metatarso-phalangical joints from SKG mice in the absence and in the presence of anti-CD4 treatment, 90 days following curdlan immunization. (D) Serum concentration of IL-6, IFN-γ, TNF and MCP-1 in naive SKG mice, SKG mice exposed to curdlan, or curlan and anti-CD4. Naive SKG mice were age matched and did not develop arthritis in the absence of curdlan immunization (no induction). The serum levels of IL-6 ($P<0.05$), IFN-γ ($P<0.01$) and MCP-1 ($P<0.01$) were significantly lower in anti-CD4 treated mice compared with animals injected with curldan in the absence of tolerizing MAbs. Differences in TNF concentration did not reach statistical significance. (E) The serum concentration of IL-10 and IL-17 in SKG mice exposed to curdlan, or curlan and anti-CD4 remained similar in the different experimental groups. Culture supernatants from Th17 cell culture were used as positive control.
doi:10.1371/journal.pone.0010558.g002

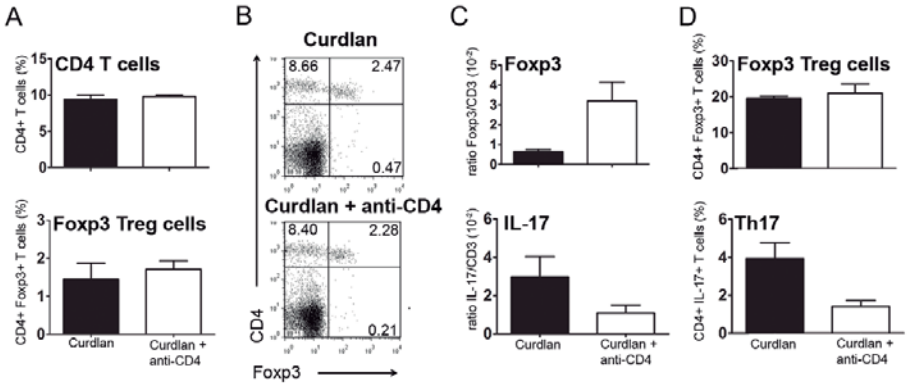

Figure 3. Anti-CD4 MAb influences the local balance of Th17/Treg cells. (A) Frequency of splenic $CD4^+$ T cells or $CD4^+Foxp3^+$ Treg cells from SKG mice exposed to curdlan, or curdlan + anti-CD4 treatment. No significant difference was observed between the two populations of animals. (B) Representative dot plots showing the frequency of splenic $CD4^+Foxp3^+$ T cells from SKG mice exposed to curdlan, or curdlan + anti-CD4 treatment. No significant difference was observed, as represented in panel A. (C) Foxp3 and IL-17 mRNA expression from the synovial membrane of SKG mice exposed to curdlan, or curdlan + anti-CD4 (mRNA expression levels were normalized to CD3 expression). (D) Frequency of $Foxp3^+$ and $IL\text{-}17^+$ T cells within draining LNs of SKG mice exposed to curdlan, or curdlan + anti-CD4.
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To address this issue we investigated whether CD4-blockade could prevent *in vitro* Th17 polarization. We used OVA-specific TCR-transgenic $CD4^+$ T cells sorted from DO11.10.$RAG^{-/-}$ mice. We stimulated these cells *in vitro*, for five days, in the presence of DCs loaded with the appropriate peptide and under culture conditions promoting optimal Th17 polarization (in presence of TGF-β, IL-6, IL-1β, and anti-IFN-γ)[30]. We observed that addition of anti-CD4 led to a significant reduction of Th17 cells (n = 6, $P<0.05$, Figure 4). In spite of the presence of cytokines that promote Th17 polarization (cytokines that are known to prevent Foxp3 induction) the addition of anti-CD4 resulted in a significant increase in the frequency of $Foxp3^+$ T cells (n = 6, $P<0.05$, Figure 4).

Non-depleting anti-CD4 induces long- term protection from autoimmune arthritis

To assess whether protection from arthritis induced with anti-CD4 treatment had a long-term effect, SKG mice initially injected with curdlan and non-depleting anti-CD4 were challenged with curdlan 60 days following the initial treatment. Animals exposed to curdlan in the presence of the putative tolerogenic anti-CD4 MAb at day 0 were protected from the induction of arthritis following curdlan administration at day 60, unlike the age-matched controls that did not receive any treatment at day 0 (n = 6, $P<0.05$, Figure 5A).

However, it should be noted, that some anti-CD4 treated mice developed mild manifestations of arthritis following curdlan challenge at day 60, although without progressing to the severe manifestations of the disease observed in control groups.

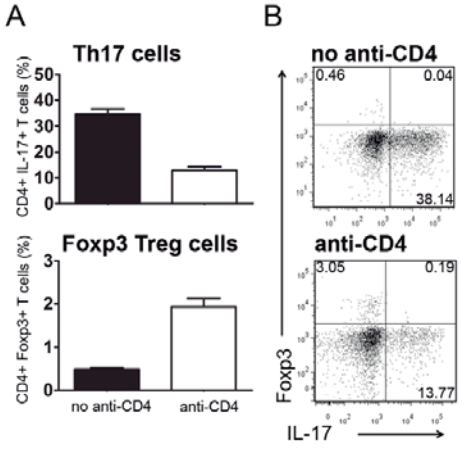

Figure 4. CD4-blockade prevents Th17 polarization. (A) Sorted TCR-transgenic cells were stimulated *in vitro* with peptide-loaded DCs under culture conditions known to preferentially polarize Th17 cells, with the addition of recombinant TGF-β, IL-6, IL-1β, and anti-IFN-γ. After 5 days of culture we observed a significant reduction of $IL\text{-}17^+$ cells in the presence of anti-CD4 (n = 6, $P<0.05$). In contrast, anti-CD4 addition led to an increased frequency of $Foxp3^+$ T cells (n = 6, $P<0.05$). (B) Representative dot plots from the two different culture conditions. An independent experiment was performed with a peptide dose of 0.3 μM with similar results.
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Non-depleting anti-CD4 prevents progression of established autoimmune arthritis

Given the fact that non-depleting anti-CD4 treatment prevents the onset of autoimmune arthritis, we tested the effectiveness of a similar course of anti-CD4 for the treatment of established arthritis in SKG mice. Female SKG mice were immunized with curdlan and when the clinical score reached 0.5 the arthritic mice were randomly included in a group treated with anti-CD4 MAb or a control group. Mice treated with non-depleting anti-CD4 showed a long-term benefit with slower disease progression and less severe clinical scores (n = 5, $P<0.05$, Figure 5B). However, remission was only achieved in a minority of the treated animals.

Anti-CD4 treated mice remain immunocompetent

A concern of immunomodulatory or tolerogenic therapeutic strategies is their long-term impact on the overall immune response. We therefore assessed the immune competence of anti-CD4-treated BALB/c mice (same genetic background as the SKG mice, but without the ZAP70 point mutation) to mount $CD4^+$ T cell-dependent immune responses towards an unrelated antigen. For this purpose, mice treated with anti-CD4 were sensitized with 20 μg OVA-alum 30 days following anti-CD4 treatment. The quantification of OVA-specific immunoglobulins in the serum was determined one week following sensitization. Our data show that the concentration of OVA-specific immunoglobulins was similar in immunized mice, regardless of previous anti-CD4 treatment, and considerable higher than in naive controls (n = 5, $P<0.05$, Figure 5C). Moreover, we confirmed the same results in SKG mice, subjected to the same protocol (n = 2, not shown). Of note, if OVA was administered at the time of anti-CD4 treatment in BALB/c mice, the animals became unable to produce OVA-specific immunoglobulins following subsequent OVA immunization (n = 6, $P<0,05$, Figure 5D), thus proving that OVA-specific IgG production is CD4-dependent, and that tolerance is only imposed over the antigens present at the time of tolerance induction.

Discussion

Our data show that a short course of non-depleting anti-CD4 can lead to long-term protection from the development of autoimmune arthritis, in a murine model of chronic disease. We have shown that our non-depleting antibody (clone YTS177) is not affecting T cells in the spleen, but seems to be preventing autoimmune arthritis, acting locally at the site of inflammation. It is likely that by targeting $CD4^+$ T cells, the pathogenic cycle of events leading to synovial inflammation and progressive joint destruction is abrogated, as other cellular players are not recruited towards the articular tissue to cause inflammation. This impact of CD4-blockade on other cell types is well illustrated by the marked decrease of pro-inflammatory cytokines produced by dendritic cells (DCs) and macrophages in anti-CD4 treated animals. In addition, given Th17 cells have been described as involved in the production of autoantibodies in experimental autoimmune arthritis [31,32], our observation that anti-CD4-treated mice do not produce RF is in line with the hypothesis that by maintaining pathogenic T cell clones under control the B cells will not receive the necessary stimuli for the production of autoantibodies. We cannot exclude, at this time, a direct effect of non-depleting anti-CD4 MAbs on innate cells expressing CD4, namely natural killer T (NKT) cells that have been reported as being able to provide "help" to B cells, and to influence autoimmune arthritis [33].

In our experiments, we found a limited efficacy for tolerance induction once the animals became overtly arthritic. Although we

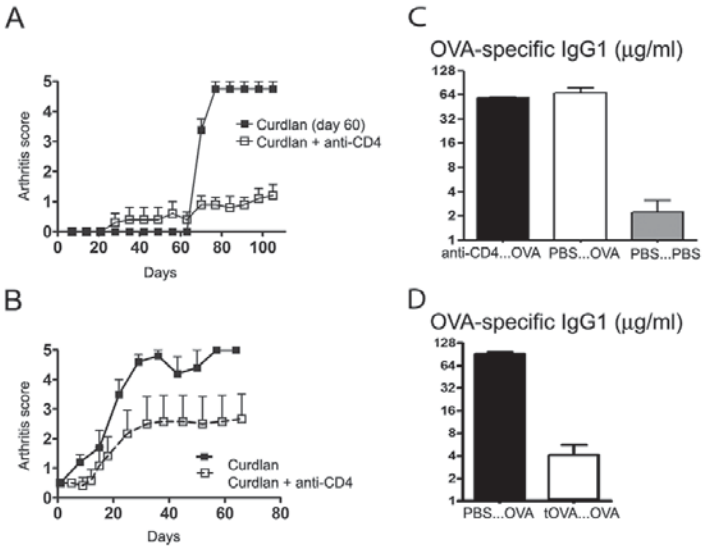

Figure 5. Long-term protective effect of non-depleting anti-CD4 treatment does not affect immune competence. (A) Female SKG mice were injected with 3 mg curdlan i.p. on day 0 and anti-CD4 on days 0, 2, and 4. Animals treated with anti-CD4 displayed long term protection from arthritis (n = 5, $P<0.05$). On day 60, anti-CD4 treated mice and age matched SKG were challenged with 3 mg curdlan i.p. Mice previously treated with anti-CD4 remained protected from the development of autoimmune arthritis (n = 5, $P<0.05$). (B) Female SKG mice were injected with 3 mg curdlan i.p. and, when the clinical score reached 0.5, some of the animals initiated treatment with three i.p. administrations of 1 mg anti-CD4 on alternate days. Control animals rapidly progressed to severe arthritis unlike the anti-CD4 treated mice (n = 6, $P<0.05$). (C) Mice were treated with non-depleting anti-CD4 30 days before immunization with OVA-alum i.p. (αCD4…OVA). One week following sensitization the serum levels of OVA-specific IgG1 were quantified, and compared with untreated (PBS…OVA) and non-immunized controls (PBS…PBS). Mice treated with anti-CD4 MAbs were competent to produce OVA-specific IgG1 to titres similar to untreated controls, and considerably higher than the non-immunized mice (n = 5, $P<0.05$). (D) OVA-specific IgG1 in mice where OVA was initially administered at the time of anti-CD4 treatment (tOVA…OVA), compared with animals that were not initially treated with anti-CD4 (PBS…OVA). Mice treated with anti-CD4 at the time of sensitization with OVA, became tolerant to the subsequent OVA immunization (n = 6, $P<0.05$).
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do not have a complete explanation for this observation, it is possible that such resistance to tolerance induction may be due to the participation of other cell types, besides $CD4^+$ T cells, at that late time. In fact, in transplantation it is known that in pre-sensitized animals a population of Asialo $GM1^+$ $CD8^+$ T cells can create a barrier for tolerance induction with MAbs [34]. Thus, it may be possible to enhance the efficacy of tolerance induction in overt arthritis with reagents targeting other cell types, namely $CD8^+$ T cells and B cells.

It remains to be established whether the long-term protection from arthritis afforded following anti-CD4 treatment, even after a new curdlan challenge at a later time, can be explained by the development of regulatory mechanisms that have been described in other animal models of anti-CD4 induced immune tolerance [28,35]. In fact, it is now established that $CD4^+$ T cell activation in the presence of TGF-β and IL-6 favors T cell conversion towards arthritogenic Th17 cells, while activation in the same environment devoid of IL-6 shifts the differentiation from Th17 towards $Foxp3^+$ Treg cells [30]. SKG autoimmune arthritis development is known to be dependent on Th17 [36].

Our data suggests that protection from arthritis induced with anti-CD4 is associated with an overall decrease of infiltrating T cells in synovial tissue and, within those T cells in the synovium, with an increase in the frequency of synovial $Foxp3^+$ Treg cells. As a consequence, the tissue is endowed with local changes that are likely to prevent the onset of arthritis directly within the local environment where inflammation would occur, even following a later exposure to curdlan at a time (day 60) where anti-CD4 MAbs are no longer present. Moreover, the reciprocal decrease of IL-17 expression at the same time that the expression of Foxp3 increases, in the joints of anti-CD4 treated mice, supports the hypothesis that indeed the balance between Treg and Th17 can determine the decision between prevention or onset of autoimmune arthritis. The observation that IL-6 decreases in the serum of anti-CD4 treated mice is also in agreement with this hypothesis. It should be noted, however, that the number of T cells is markedly reduced in the synovium of treated mice, as can be seen in the histological sections (and confirmed with greater CD3 expression in the synovia by RT-PCR – thus the need to use CD3, rather than a housekeeping gene, to normalize our gene expression studies). It was recently reported that IL-17A can be produced by mast cells in rheumatoid arthritis synovia [37]. Although we did not investigate this possible source of IL-17, the overall quantification of IL-17 transcripts was significantly reduced in anti-CD4 treated animals, where T cells were also less abundant. As a consequence, the shift in the Treg/Th17 balance in the synovial tissue is likely to be also influenced by tissue accessibility to different types of effector T cells.

We have also shown that CD4-blockade can directly inhibit T cell polarization towards an IL-17-producing Th17 phenotype, even when the most appropriate cytokine environment is provided. In addition, our data show that even in the presence

of those cytokines known to inhibit Foxp3 induction (namely IL-6), CD4-blockade can facilitate the peripheral conversion of $Foxp3^+$ Treg cells. Taken together, our data complements previous studies on the mechanism of tolerance induction in transplantation with anti-CD4 (where peripheral induction of $Foxp3^+$ Treg cells have been shown critical), by demonstrating that CD4-blockade can also lead to a direct inhibition of Th17 polarization – a critical factor for the arthritis pathogenesis, namely in SKG mice [36].

Given the essential role of $CD4^+$ T cells in the pathogenesis of RA, both directly and by recruiting and activating other participating cell types (such as B cells, DCs and macrophages), the therapeutic targeting of $CD4^+$ lymphocytes has been extensively pursued [38]. Depleting and non-depleting anti-CD4 MAbs have been tested in several animal models of autoimmune arthritis [39,40,41,42], and in clinical trials with RA patients [43,44,45]. In spite of promising results in pre-clinical studies, the therapeutic effectiveness of anti-CD4 in clinical trials was modest and short-term, possibly due to transient immunosuppression and not tolerance. In retrospect, those unimpressive results are not too surprising due to technical details related with dosing and MAb characteristics. In fact the immunogenicity of the mouse or chimeric MAb used was well documented as leading to their rapid clearance and consequent loss of efficient CD4 blockade [46]. The reduction of the number of $CD4^+$ T cells was also associated with a concern of possible increased susceptibility to infection.

Our data show that non-depleting anti-CD4 can be effective in preventing chronic autoimmune arthritis while preserving immune competence, as treated mice remain able to mount a CD4-dependent immune response against a different antigen (OVA). OVA immunization is a well established protocol for the induction of CD4-dependent production of OVA-specific immunoglobulins. Moreover, administration of anti-CD4 together with OVA prevents the effectiveness of subsequent immunizations with the same antigen. Taken together, these data suggest that anti-CD4 prevents immune responses towards antigens present at the time of treatment, without hampering immune responses to unrelated antigens introduced in the organism at a later time, therefore preserving immunocompetence. It should be noted that these types of studies, on the long-term immunocompetence of treated mice have been difficult to perform in animal models that, unlike SKG mice, do not develop a chronic form of arthritis.

In summary, we show that therapeutic strategies leading to synovial accumulation of Treg cells and reduction of Th17 are capable of protecting from the onset of autoimmune arthritis, as well as to prevent long-term disease progression, without leading to overall immune suppression.

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Author Contributions

Conceived and designed the experiments: JD JEF LG. Performed the experiments: JD AAD VO. Analyzed the data: JD AAD VO JEF LG. Wrote the paper: JD JEF LG.

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