

# A T cell receptor transgenic model of severe, spontaneous organ-specific autoimmunity

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The development of mouse models of human organ-specific autoimmune diseases has been hampered by the need to immunize mice with autoantigens in potent adjuvants. Even autoantigen-specific T cell receptor transgenic models of autoimmunity have proven to be complex as the transgenic mice frequently fail to develop disease spontaneously. We have isolated a CD4<sup>+</sup> T cell clone (TXA23) that recognizes the gastric parietal cell antigen, H/K ATPase  $\alpha$ -chain<sub>630–641</sub>, from a mouse with autoimmune gastritis that developed after thymectomy on day 3 of life. The T cell receptor  $\alpha$  and  $\beta$  genes from this clone were used to generate A23 transgenic mice. All A23 transgenic animals spontaneously developed severe autoimmune gastritis, and evidence of disease was detected as early as day 10 of life. Gastritis could be transferred to immunocompromised mice with a limited number of transgenic thymocytes (10<sup>3</sup>), but as many as 10<sup>7</sup> induced only mild disease in wild-type animals. Due to the complete penetrance of spontaneous disease, identity of the auto-antigen, susceptibility to immunoregulation, and close relation to autoimmune gastritis in man, A23 transgenic mice represent a unique CD4<sup>+</sup> T cell-mediated disease model for understanding the multiple factors regulating organ-specific autoimmunity.

**Key words:** Gastritis / H/K ATPase / Transgenic / Autoimmune model

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## 1 Introduction

Organ-specific autoimmunity represents a group of diseases resulting from a dysregulation of B and T cell immune function leading to the insidious and progressive destruction of a target organ. To date, the most informative and best-studied animal models of organ-specific autoimmunity have employed autoantigen-specific TCR transgenic (Tg) animals. Several Tg TCR models have been developed from T cell clones derived from genetically susceptible nonobese diabetic (NOD) mice that spontaneously develop insulin-dependent diabetes mellitus (IDDM) [1, 2]. However, the incidence of disease in these NOD Tg animals is only modestly increased and the target antigen has proven difficult to define. Most other autoantigen-specific Tg TCR animals have been generated by potentially selecting a rare path-

ogenic TCR following hyperimmunization with the target autoantigen in Freund's complete adjuvant [3–5]. In the majority of the resultant Tg mouse strains, however, immunization with the target antigen is still required to elicit disease and disease penetrance is not complete.

An important alternate model system for the study of organ-specific autoimmunity has been thymectomy of mice on day 3 of life (d3Tx), resulting in strain-dependent development of diseases such as gastritis, oophoritis, orchitis, thyroiditis, pancreatitis, and prostatitis [6–8]. Several studies indicate that the important regulatory cells eliminated by d3Tx are CD4<sup>+</sup>CD25<sup>+</sup> T cells that develop late in ontogeny and are deficient in the peripheral lymphoid tissues of the d3Tx mouse [9–11]. CD4<sup>+</sup>CD25<sup>+</sup> T cells have also recently been shown to play critical roles in the development of inflammatory bowel disease [12] and IDDM in the NOD mouse [13].

We have focused our efforts on autoimmune gastritis (AIG) as a model to further elucidate the pathophysiology of organ-specific autoimmunity and the role of regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells *in vivo*. Following d3Tx, 60% of BALB/c mice develop AIG within 3 months, which closely resembles pernicious anemia in man [14, 15]. Suri-Payer et al. [16] isolated several H/K ATPase

[1 21792]

**Abbreviations:** **AIG:** Autoimmune gastritis **Tg:** Transgenic **d3Tx:** Thymectomy on day 3 of life **NOD:** Nonobese diabetic **IDDM:** Insulin-dependent diabetes mellitus **SP:** Single positive **WT:** Wild type **PCAb:** Parietal cell Ab



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### 2.3 Selection of T cell populations in A23 Tg mice

In screening for transgene positive animals by flow cytometry, we observed that the percentage of CD4<sup>+</sup> cells in peripheral blood was lower in animals expressing the rearranged TCR (data not shown). Since mRNA for the H/K ATPase  $\alpha$ -chain can be detected in the thymus as early as day 18 of gestation (C. Piccirillo, unpublished results), it was possible that  $\alpha$ -chain-derived peptides in the thymus lead to negative selection of the A23 Tg TCR-

expressing T cells. To evaluate the thymic selection of the A23 Tg T cells, thymocytes were harvested and analyzed for CD4, CD8 and Tg TCR expression. There was no difference in the size of the thymus or the total number of cells recovered from A23 Tg and non-Tg littermates (data not shown). When the A23 Tg TCR was expressed in H-2<sup>d</sup> animals, marked skewing toward the CD4 single-positive (SP) phenotype was observed. In the H-2<sup>d</sup> animal, CD4 SP cells represented 43% of thymocytes, as compared to 9.6% in the non-Tg littermate (Fig. 2A and B). A high percentage (90%) of the CD4 SP cells, in the H-2<sup>d</sup> animal, expressed the Tg TCR (Fig. 2B, right histogram). Cells expressing the A23 Tg TCR were also detected in H-2<sup>b</sup> mice, with over 80% of CD4 SP cells expressing the rearranged TCR (Fig. 2C); however, skewing toward the CD4 SP population was not apparent in A23 Tg H-2<sup>b</sup> thymocytes (3.1% vs. 9.6% in non-Tg littermate control, Fig. 2A). In the periphery of A23 Tg animals, there is only a slight increase in the CD4/CD8 ratio in both the spleen (Fig. 2D and E) and peripheral LN (data not shown) as compared to non-Tg littermate. TCR transgene expression in the periphery is similar to that seen in the thymus, 80–95% of CD4 SP cells (data not shown).

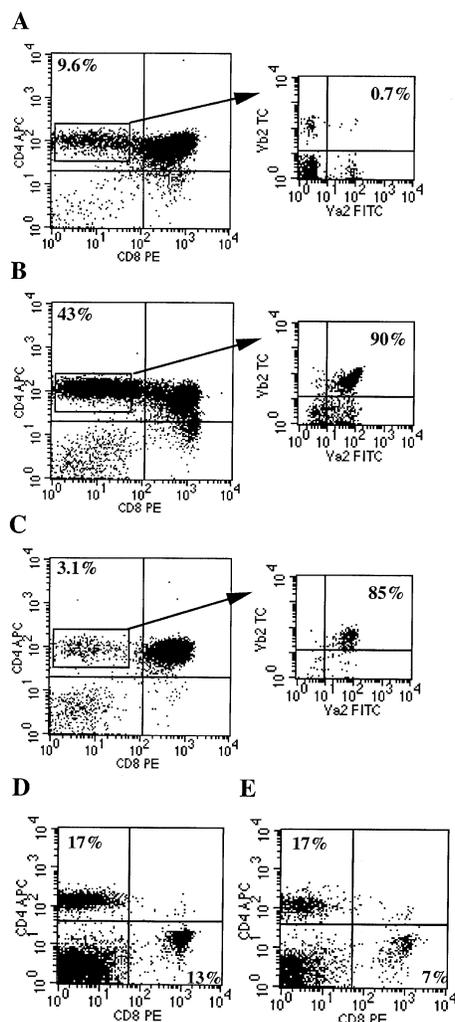


Fig. 2. Expression of A23 transgene in thymocytes. The thymus was harvested from 2-month-old non-Tg (A), A23 Tg H-2<sup>d</sup> (B) and A23 Tg H-2<sup>b</sup> (C) littermates. Cells were analyzed for CD4 and CD8 expression. CD4 SP cells were further analyzed for V $\alpha$ 2 and V $\beta$ 2 transgene surface expression. Splenocytes from non-Tg (D) and A23 Tg H-2<sup>d</sup> (E) were also analyzed for CD4, CD8, and transgene expression by flow cytometry.

### 2.4 Spontaneous development of AIG in A23 Tg mice

The TxA23 clone was shown to cause AIG in immunodeficient, but not WT, recipient animals upon adoptive transfer of activated T cell blasts. It was of interest whether AIG would develop in immunocompetent animals expressing the rearranged TCR from TxA23. Upon histological analysis of unmanipulated H-2<sup>d</sup> A23 Tg animals, all were found to have severe lymphocytic infiltration into the gastric mucosa with varying degrees of parietal cell loss (Fig. 3B). The pathology of the gastric mucosa of a 2-month-old A23 Tg mouse with an H-2<sup>d</sup> background is similar to that of an AIG<sup>+</sup> d3Tx mouse. Both are characterized by lymphocytic infiltration into the mucosa and sub-mucosa, with marked depletion of parietal and chief cells. Replacement of the normal gastric pit architecture with abundant mucus-producing cells is also evident (Fig. 3B, arrows). An A23 Tg H-2<sup>b</sup> mouse and non-Tg littermate control are shown for comparison, both displaying normal gastric mucosa (Fig. 3C and A, respectively).

### 2.5 Expression of activation markers in peripheral lymphoid tissues

Gastric LN of AIG<sup>+</sup> A23 Tg animals were extremely enlarged, as compared to the barely detectable gastric LN in WT animals. Gastric LN cells of A23 Tg animals

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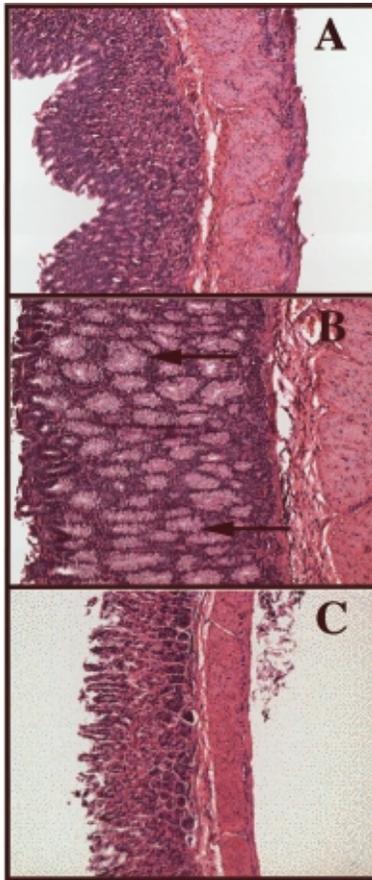


Fig. 3. A23 Tg H-2<sup>d</sup> transgenic mice develop spontaneous and severe autoimmune gastritis. Stomachs were harvested from 2-month-old Non-Tg (A), A23 Tg H-2<sup>d</sup> (B) and A23 Tg H-2<sup>b</sup> (C) littermates. The sections were stained with H&E and analyzed for gastric pathology by microscopic observation.  $\times 10$  magnification.

were composed of 45% Tg T cells (data not shown), with the majority expressing the early activation antigen, CD69 (Fig. 4A, bottom right). Although the CD4<sup>+</sup> population in peripheral LN was markedly enhanced (>90%) for the percentage of Tg TCR-expressing cells, only a slightly increased level of CD69 expression was observed as compared to the non-Tg littermates, 13% vs. 7%, respectively (Fig. 4A, left histograms). It is, therefore, likely that localized activation of the A23 Tg T cells is taking place in the gastric LN, but we are unable to distinguish whether the up-regulation of CD69 is due to continuous stimulation of the same cells in the target organ or stimulation of waves of new Tg T cells homing to the gastric LN. Curiously, although CD69 expression was up-regulated, the level of IL-2R $\alpha$  chain (CD25), also considered to be a marker of T cell activation, did not markedly differ among the LN populations analyzed (Fig. 4B). As a control, expression of these two activation

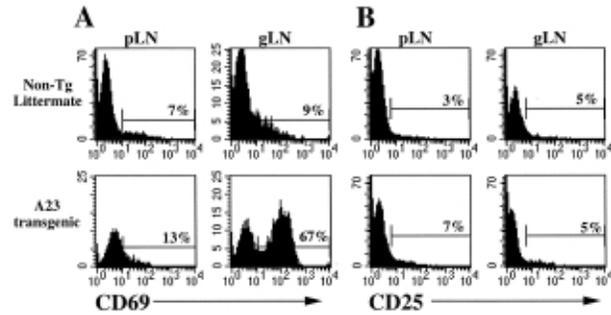


Fig. 4. Activation of A23 Tg T cells at the site of the target organ. Peripheral (p) and gastric (g) LN were harvested from non-Tg (top panels) and A23 Tg H-2<sup>d</sup> (bottom panels) littermates. V $\alpha$ 2<sup>+</sup>V $\beta$ 2<sup>+</sup> cells were analyzed for CD69 (A) and CD25 (B) expression in the peripheral (inguinal, axillary and mesenteric) and gastric LN cells.

markers on A23 Tg cells of H-2<sup>b</sup> animals did not differ between mesenteric and gastric LN (data not shown), indicating that activation requires not only expression of the Tg TCR, but also the presence of I-A<sup>d</sup>. In addition, *in vitro* analysis of A23 Tg T cells confirmed specificity of the T cell for H/K ATPase  $\alpha$ -chain<sub>630-641</sub> in the presence of I-A<sup>d</sup> by both proliferation and effector cytokine production (data not shown).

## 2.6 Evidence of early disease induction

Since both the A23 Tg TCR and H/K ATPase  $\alpha$ -chain are expressed early in ontogeny, we were curious to see how early signs of inflammation could be identified in the target organ. Litters of various ages (2, 7, 10, 14, 21 days old) were analyzed for the presence of A23 Tg TCR, gastric infiltration and CD69 expression on Tg T cells. As early as day 10 of life, signs of gastric pathology and enlarged gastric LN were evident (Fig. 5A, lower panel). A23 Tg animals had large isolated infiltrations into the gastric mucosa at day 10. Non-Tg littermates had no evidence of lymphocytic infiltration or enlarged gastric LN at any age tested (Fig. 5A, top panel).

Analysis of T cell populations in the gastric LN at day 10, as compared to other peripheral lymphoid tissues, shows a striking abundance of A23 Tg T cells. Flow cytometry of the gastric LN shows that over 40% of total cells recovered expressed the A23 Tg TCR (Fig. 5B, bottom dot plot). The mesenteric LN of A23 Tg animals at day 10 showed only 1–2% A23 Tg T cells present (data not shown). This suggests that migration to, or proliferation at, the target organ is active at this early time. We further analyzed the A23 Tg T cells in the gastric LN for signs of activation. Greater than 50% of Tg T cells in the gastric LN had up-regulated CD69 on the cell surface

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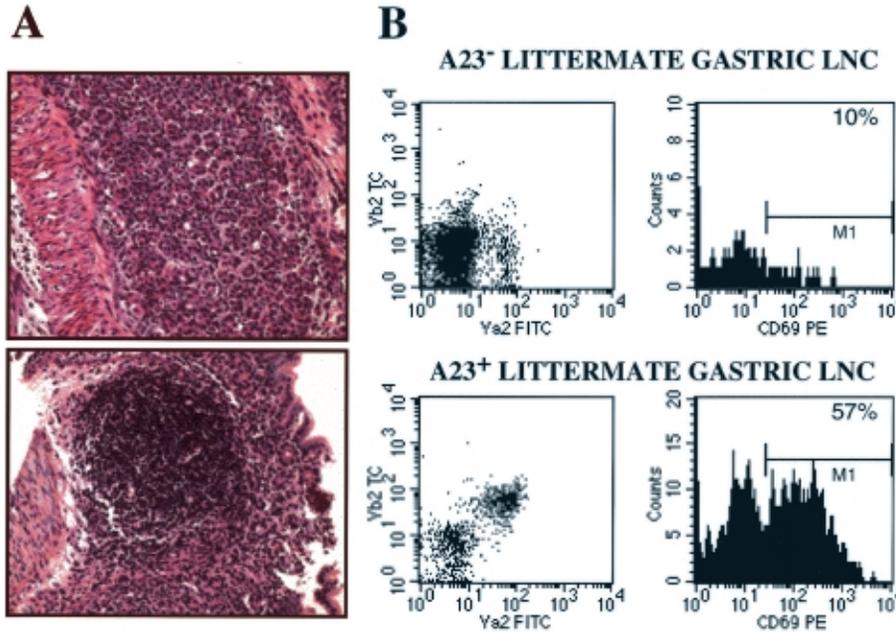


Fig. 5. Evidence of gastric pathology by day 10 of life. Stomachs and gastric LN were harvested from 10-day-old pups of an A23 Tg H-2<sup>d</sup> transgenic mouse. (A) Stomachs were stained with H&E and analyzed for gastric pathology ( $\times 10$  magnification). (B) Gastric LN cells expressing V $\alpha$ 2 and V $\beta$ 2 were analyzed for CD69 expression.

(Fig. 5B, bottom histogram). In contrast, as in the adult, CD69 expression was minimal in Tg T cells in the peripheral LN (data not shown), as well as in gastric LN of non-Tg littermate controls (Fig. 5B, upper histogram).

### 2.7 Thymocytes from A23 Tg mice induce AIG in BALB/c nu/nu mice

Previous studies of TxA23 cells have indicated that activated cells transfer AIG when introduced into immunodeficient hosts [16], but not WT, animals. The ability to confer disease might be a function of the antigen specificity of the TCR expressed, as well as the state of differentiation of the T cell population. The T cells from A23 Tg mice might represent different stages of differentiation compared to the clone TxA23; therefore, it was of interest to determine the ability of various populations of A23 Tg T cells to efficiently transfer AIG to immunodeficient or immunocompetent mice.

Thymocytes from A23 Tg animals were introduced intravenously into BALB/c nu/nu and (C57BL/6 $\times$ BALB/c)F1 mice in amounts ranging from 10 to 10<sup>7</sup> thymocytes. Analysis of both anti-parietal cell Ab (PCAb) (data not shown) and gastric pathology at 6 weeks post transfer revealed that as few as 10<sup>3</sup> thymocytes could transfer AIG to a BALB/c nu/nu mouse (Fig. 6, left panel). A23 Tg

T cells from both the peripheral and gastric LN were also able to induce AIG (data not shown). In contrast, more than 10<sup>7</sup> A23 Tg thymocytes were required to induce moderate AIG in WT (C57BL/6 $\times$ BALB/c)F1 recipients (Fig. 6, right panel), indicating an ability of immunocompetent animals to control the disease-inducing cells. Transfer studies using naive A23 Tg T cells could provide a unique tool to evaluate the initial activation of AIG as well as the requirements for suppression.

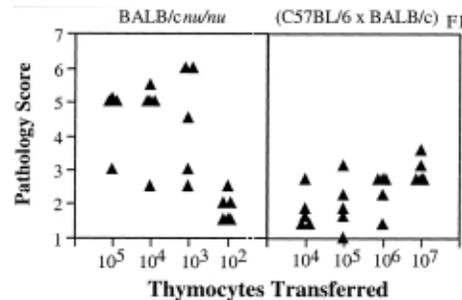


Fig. 6. A23 Tg thymocytes transfer AIG to immunocompromised mice. Thymocytes from 6-week-old A23 Tg H-2<sup>d</sup> animals were harvested and injected i.v. into BALB/c nu/nu or (C57BL/6 $\times$ BALB/c)F1 mice at various cell concentrations. At 4 weeks post transfer, stomachs were analyzed for gastric pathology.

Figure 5 = 4c

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## 2.8 CD4<sup>+</sup>CD25<sup>+</sup> suppressor T cells are present in the thymus of A23 Tg mice

One explanation for the high incidence of spontaneous disease in A23 Tg mice is that expression of the Tg TCR inhibits or delays the development of the population of CD4<sup>+</sup>CD25<sup>+</sup> suppressor cells [9]. To determine whether CD4<sup>+</sup>CD25<sup>+</sup> suppressor cells exist in A23 Tg animals, CD4<sup>+</sup>CD25<sup>+</sup> cells were sorted from the thymus of a 3–6-week-old A23 Tg H-2<sup>d</sup> animal and tested *in vitro* for their ability to suppress proliferation of WT CD4<sup>+</sup> cells in response to anti-CD3 stimulation. CD4<sup>+</sup> CD25<sup>+</sup> thymocytes from A23 Tg animals were as efficient as WT CD4<sup>+</sup>CD25<sup>+</sup> in their ability to suppress proliferation to anti-CD3, 84% vs. 88% suppression, respectively, at the highest number of regulatory T cells (Fig. 7). Although this result indicates that CD4<sup>+</sup>CD25<sup>+</sup> suppressors are present in these young adult animals, it does not exclude the possibility that their development is delayed and that the disease process had already been initiated prior to their emergence from the thymus.

## 3 Discussion

We describe here a unique model for the study of CD4<sup>+</sup> T cell-mediated, organ-specific autoimmunity. The T cell clone used as the donor of the TCR for the Tg model of AIG was derived from an animal that developed AIG following d3Tx [16], which prevents emigration of immunoregulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells. Thus, the pathophysiol-

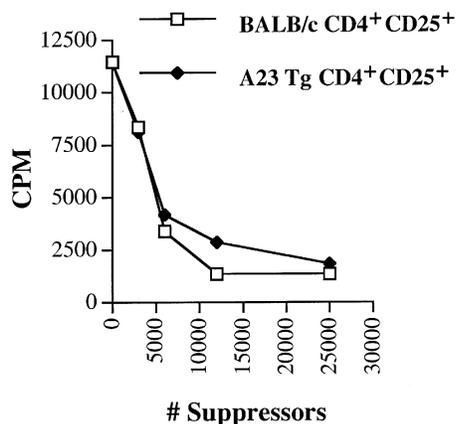


Fig. 7. CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells are present in the adult A23 Tg mouse. CD4<sup>+</sup> CD25<sup>+</sup> cells were sorted from the thymus of 3–6-week-old A23 Tg mice (closed diamonds) and compared to WT BALB/c CD4<sup>+</sup>CD25<sup>+</sup> cells (open squares) in a standard *in vitro* suppression assay. Proliferation of responding T cells was measured by [<sup>3</sup>H]thymidine incorporation for the last 12 h of a 72-h culture.

ogy of this model may be quite distinct from other models of organ-specific autoimmunity, particularly those based on immunization of mice with autoantigens. One important difference between A23 Tg mice and other Tg models of CD4<sup>+</sup> T cell-mediated autoimmunity is the incidence of spontaneous disease. Several Tg TCR models of IDDM have been developed from cells isolated from NOD mice. The most widely studied model, the BDC2.5 mouse [1], used TCR genes derived from a CD4<sup>+</sup> T cell that had been naturally primed to an islet cell antigen. This Tg mouse, however, does not develop IDDM at an accelerated rate compared to a non-Tg NOD mouse, and the target antigen recognized by the TCR has not been defined. The 4.1 mouse [2], also derived from an NOD, did develop IDDM at an accelerated rate, but the  $\beta$ -cell antigen seen by this Tg TCR remains undefined. When either of these Tg TCR animals are crossed to mice lacking an endogenous T cell repertoire, the disease onset and incidence are dramatically increased suggesting that regulatory T cells modulate the development of IDDM.

Other CD4<sup>+</sup> Tg TCR models of autoimmunity were developed using the TCR from autoreactive T cells derived from animals immunized with autoantigens in adjuvant. Interestingly, these Tg mice develop spontaneous autoimmune disease at a very low rate, often requiring immunization with the autoantigen to induce clinical symptoms. In the best-studied Tg TCR model of EAE, disease resistance is shown to be due to a population of CD4<sup>+</sup> TCR  $\alpha\beta^+$  immunoregulatory T cells, but the relationship of these regulatory T cells to the CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells remains to be defined [18, 19]. In other Tg TCR EAE models, disease can be induced by maintaining Tg mice in a “dirty” environment or by the injection of pertussis toxin [3]. Recently, several Tg TCR lines have been generated from T cell clones derived from animals that were immunized with proteolipid protein [20]. These Tg models of EAE, unlike those previously studied, develop fulminant spontaneous EAE, with incidence reaching 80% in some instances. However, mice developing severe spontaneous EAE are difficult to maintain and breed.

A model for arthritis, the K/BxN TCR Tg mouse, has been described where the antigen is also known and disease occurs spontaneously in 100% of observed mice [21]. These Tg animals require the KRN TCR, H-2A<sup>g7</sup> and production of autoreactive Ig by activated B cells [22]. Arthritis can be transferred to immunodeficient hosts by either splenocytes or sera from K/BxN Tg mice. Although these animals provide an excellent model for understanding the pathology of arthritis, the requirement for both autoreactive T and B lymphocyte suggests a more complex model than the A23 Tg mouse for investigation of an organ-specific autoimmune disease.

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The difference between spontaneous and induced models of CD4<sup>+</sup> T cell-mediated autoimmunity is best illustrated by a comparison of the A23 Tg model of AIG with a second Tg TCR model of AIG developed by Alderuccio et al. [23]. Studies by this group suggest that it is the H/K ATPase  $\beta$ -chain that plays an important role in the initiation of AIG post-d3Tx [24]. T cells specific for the  $\beta$ -chain, however, have only been identified following immunization with the intact H/K ATPase or purified  $\beta$ -chain preparations [25]. An anti-H/K ATPase  $\beta$ -chain-specific TCR was isolated from a T cell hybridoma derived by immunization of BALB/c mice with the H/K ATPase  $\beta$ -chain<sub>253-277</sub> peptide. In contrast to the A23 Tg mice, only a minority (20%) of the anti- $\beta$ -chain Tg TCR mice developed AIG. It is possible that the difference between the anti- $\beta$ -chain Tg TCR mice and A23 Tg mice may be due to differences in the affinity of the respective TCR for their target antigens. The A23 Tg TCR may be of high affinity, while the anti- $\beta$  chain TCR may be of relatively low affinity. T cells expressing this latter receptor could, therefore, only be isolated following their expansion induced by immunization and are only of moderate pathogenicity. Other differences also exist between the two anti-H/K ATPase of TCR Tg animals. The  $\beta$ -chain-specific Tg TCR T cells appear to be inefficiently selected in the thymus as the thymus size of the Tg mice was small and the percentage of SP CD4 cells was decreased from approximately 6.7% in non-Tg littermates to 0.8%. Although the majority of peripheral CD4<sup>+</sup> T cells do express the Tg TCR, perhaps the inefficient emigration of these cells from the thymus could also allow for control by the CD4<sup>+</sup>CD25<sup>+</sup> suppressor cells.

An appealing reason for choosing the AIG model is that immunoregulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells appear to play a major role in determining susceptibility or resistance to this disease. Although CD4<sup>+</sup>CD25<sup>+</sup> T cells have now been shown to play important roles in the control of several models [12, 13] of organ-specific autoimmunity in the mouse, in addition to those observed after 3dTx, their role in the normal human immune response and in human autoimmune diseases remains to be defined. One important question to be more fully addressed about the A23 Tg model of AIG is the susceptibility of the Tg T cells to suppression by the CD4<sup>+</sup>CD25<sup>+</sup> cells. We are able to readily transfer AIG with as few as 10<sup>3</sup> thymocytes from Tg mice into *nu/nu* recipients, while 10<sup>7</sup> cells produced only slight disease upon transfer to normal recipients. Although this result is consistent with CD4<sup>+</sup>CD25<sup>+</sup>-mediated regulation in the normal recipient, it is possible that a component of the enhanced susceptibility of *nu/nu* mice is due to the nonspecific expansion of T cells following transfer to a lymphopenic environment [26]. Given that Tg cells are susceptible to CD4<sup>+</sup>CD25<sup>+</sup>-mediated immunoregulation, the A23 Tg

model will allow us in addition to address many important questions regarding suppressor function *in vivo*.

Functioning CD4<sup>+</sup>CD25<sup>+</sup> T cells could be detected in the thymus of A23 Tg young adult animals at a frequency similar to that reported in normal BALB/c mice; however, all of A23 Tg mice developed spontaneous severe AIG, indicating a failure of suppressor cell function *in vivo*. One simple explanation is that the tremendous excess of H/K ATPase  $\alpha$ -chain specific T cells disrupts the delicate balance between effector and immunoregulatory cells. However, as noted above, in other CD4<sup>+</sup> Tg T cell models of autoimmunity, such as EAE, immunoregulatory cells function normally, even in the presence of an excess of effectors, as disease incidence still remains low. That there is an important factor that may render mice expressing a TCR specific for an autoantigen susceptible to autoimmune disease is shown by the observation that expression of any Tg TCR  $\alpha$ -chain resulted in delayed development of the CD4<sup>+</sup>CD25<sup>+</sup> population, leading to AIG in up to 60% of mice on a susceptible background [9]. Since autoimmune disease does not spontaneously develop in most other CD4<sup>+</sup> Tg TCR models of autoimmunity, delayed development of regulatory T cells cannot be the sole mechanism for autoimmune disease in the A23 Tg mouse.

Collectively, there appear to be several unique differences between the recognition of the H/K ATPase and other autoantigens. First, although mRNA for the H/K ATPase  $\alpha$ -chain can be readily detected in the thymus, it is unclear if central tolerance to any component of the H/K ATPase specific repertoire is ever generated. Secondly, studies using d3Tx to induce AIG have shown that control of disease must be achieved early, within 10 days post thymectomy [10]. Lastly, expression of the H/K ATPase can be detected as early as day 18 of gestation and it represents one of the most abundant proteins in gastric parietal cells. Furthermore, the accessibility of the H/K ATPase to naive T cells may be enhanced by expression in a cell type that is rapidly turning over [27]. Macrophages or specialized dendritic cells in the gastric mucosa may take up the debris created by the death of gastric parietal cells and migrate to the draining LN, where the antigen can be transferred to dendritic cells for presentation to specific T cells [28]. All of these factors likely contribute to the development of disease in the A23 Tg prior to the delayed emigration from the thymus of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells. These results also suggest that not all tissue-specific autoantigens are treated equivalently by the immune system. Peripheral factors such as time of expression, level of expression, and accessibility to the lymphoid system may prove to be major determinants of the susceptibility of a given organ to autoimmune disease.

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**4 Materials and methods****4.1 Mice and reagents**

BALB/c, C57BL/6, (C57BL/6×BALB/c)F1 and BALB/c *nu/nu* mice were purchased from NCI/Frederick Animal facility and maintained under SPF conditions. Tg mice generated in (C57BL/6×BALB/c)F1 were backcrossed to both the BALB/c and C57BL/6 backgrounds.

Antibodies used in flow cytometry specific for CD4 (GK1.5), V $\alpha$ 2 (B20.1), V $\beta$ 2 (B20.6), H-2K<sup>b</sup> (AF6–88.5), H-2D<sup>d</sup> (34-2-12), CD8 (53–6.7), CD25 (7D4 and PC61), CD69 (H1.2F3), and Fc Block (CD216/CD32; 2.4G2), were purchased from PharMingen (San Diego, CA). Anti-CD4-Tri-Color (TC) (CT-CD4) and streptavidin-TC was purchased from Caltag (Burlingame, CA). Streptavidin-PE was purchased from Southern Biotechnologies Associates (Birmingham, AL).

**4.2 DNA constructs and generation of A23 Tg mice**

Total RNA was extracted from 4×10<sup>6</sup> TxA23 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. cDNA was transcribed from 2  $\mu$ g total RNA in a 20- $\mu$ l reaction using oligo(dT) primer, first strand cDNA synthesis reagents, and SUPERScript II reverse transcriptase (Gibco/Life Technologies, Gaithersburg, MD). Full-length TCR  $\alpha$ -chain cDNA, beginning with the initiation ATG codon of the leader and ending with the termination codon at the 3' end of C $\alpha$  was amplified from total cDNA by PCR using primer (5'CCGAATTCATGGACAAGATCCTGACAGCATCGTTT) and reverse primers (5'TCCCCCGGGTTATCAACTGGACCACAGCCTCAGCGTCATGAG), incorporating EcoRI and SmaI sites (underlined), respectively. The amplified DNA was ligated to the TA cloning vector pCR2.1 (Invitrogen) and plasmid DNA obtained from transformed colonies was sequenced (Applied Biosystems Sequencer, Model 377) to confirm fidelity of the PCR. Insert DNA was excised from pCR2.1 using EcoRI and SmaI and ligated to the corresponding sites of a CD2-based expression vector [29], kindly provided by Dr. D. Koussis (National Institute of Medical Research, London). Prokaryotic sequences were removed by NotI/SalI digestion and gel purification prior to injection into (C57BL/6×BALB/c)F1 blastocysts.

An alternate vector using an MHC class I promoter [30], kindly provided by Dr. H. Pircher (University of Freiburg, Freiburg) was used for expression of TCR  $\beta$  cDNA. Full-length TCR  $\beta$  cDNA, beginning with the leader and ending with the stop codon at the 3' end of C $\beta$  was amplified by PCR on total cDNA, obtained as above, using forward (5'-ACGC-GTCGACCCCAGCCATGTGGCAGTTTTGCATTCTGTGCCTC) and reverse primers (5'-TCCCCCGGGTTATCAGGAATTTT-TTTTCTTGACCATGGCCATCAGCACCAGGCCACTGACC). The forward primer included a SalI site (underlined) and a consensus Kozak sequence (italicized) immediately prior to

the ATG start codon. The amplified fragment was ligated to pCR2.1 and plasmid DNA from transformants was sequenced. The V $\beta$  cDNA was excised by SalI/BamHI digestion and ligated to the corresponding sites in the pHSE3' vector [30]. Prokaryotic sequences were removed by XhoI digestion and gel purification prior to blastocyst injection. DNA sequences were analyzed with the DNA plot analyses of the ImMunoGeneTics database (<http://imgt.cines.fr:8104/dnaplot/>). Transgenic generation and animal husbandry were accomplished at the NIAID transgenic and knockout facility (FCRDC, Frederick, MD) under SPF conditions.

At the time of weaning, TxA23 Tg TCR mice were identified by flow cytometry of peripheral white blood cells. Twenty microliters of heparinized blood were lysed in 200  $\mu$ l ACK lysis buffer (Biofluids, BioSource International, Rockville, MD) and washed once in staining buffer (PBS containing 1% FCS and 0.01% NaN<sub>3</sub>). Ten microliters of appropriately diluted FC Block and anti-V $\beta$ 2 biotin were added and incubated at 4°C. The cells were washed and 10  $\mu$ l anti-CD4 TC, anti-V $\alpha$ 2 FITC and streptavidin-PE were added and incubated at 4°C. Cells were washed and analyzed by flow cytometry of live lymphocytes using CellQuest software (BD Biosciences, San Jose, CA).

**4.3 Analysis of AIG**

At various time points, offspring from A23 Tg lines were bled for sera and analyzed for the presence of PCAb by immunofluorescence on cryo-sections of normal BALB/c stomach as previously described [31]. For histological analysis of Tg mice and BALB/c *nu/nu* mice, stomachs were fixed in 4% paraformaldehyde and sections were prepared and stained with hematoxylin and eosin (H&E) by American Histolabs. Pathology was evaluated as described previously [11] using a disease score from normal gastric mucosa (0–1) to severe AIG (6).

**4.4 Flow cytometry**

Lymphoid tissues were harvested from A23 Tg and non-Tg littermates at various ages. Tissues were processed into single-cell suspensions and stained for the presence of Tg TCR by first staining with biotinylated anti-V $\beta$ 2, followed by streptavidin-PE, anti-V $\alpha$ 2 FITC and anti-CD4 TC. Thymocytes and splenocytes were also multi-stained with anti-V $\beta$ 2 biotin, followed by streptavidin-TC, anti-V $\alpha$ 2-FITC, anti-CD4-APC and anti-CD8-PE. In addition, peripheral (inguinal, axillary, or mesenteric) LN cells were analyzed for CD69 and CD25 expression using anti-CD69- and anti-CD25-PE.

**4.5 T cell suppression assay**

CD4<sup>+</sup>CD25<sup>+</sup> cells were sorted from the thymus of 3–6-week-old A23 Tg mice. Thymocytes were harvested and live cells were recovered after density centrifugation (Lympholyte M,

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Cedarlane Laboratories Limited, Hornby, Ontario, Canada). Cells were stained with anti-CD4-TC, anti-CD8-FITC, and anti-CD25-PE and sorted for CD4<sup>+</sup>CD25<sup>+</sup>CD8<sup>-</sup> cells on a BD FACS Vantage SE. Suppression assays and magnetic bead purification of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells from WT BALB/c mice were described previously [32]. CD4<sup>+</sup>CD25<sup>-</sup> T cells (0.5 × 10<sup>6</sup>/ml) were stimulated with T-depleted splenocytes (0.5 × 10<sup>6</sup>/ml) and 0.5 µg/ml anti-CD3 (2C11, PharMingen) in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells at suppressor:responder ratios of 0:1, 1:2, 1:4, 1:8 and 1:16. Proliferation was measured by adding [<sup>3</sup>H]thymidine (1 µCi/well) for the final 12–16 h of a 72-h culture.

## 4.6 Transfer of AIG

Thymocytes, gastric and peripheral LN cells were harvested from 6–8-week-old A23 Tg mice. Following removal of contaminating erythrocytes by ACK lysis, cells were resuspended in PBS at a density of 5 × 10<sup>3</sup>–5 × 10<sup>7</sup> cells/ml. Cells (200 µl) were injected i.v. into 6–8-week-old BALB/c *nu/nu* or (C57BL/6 × BALB/c)F1 mice. Animals were analyzed for AIG by detection of anti-PCAb in sera 4–6 weeks post transfer. Stomachs were also analyzed for pathology at various times as described earlier.

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