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Autoimmune Gastritis Is a Well-Defined Autoimmune Disease Model for the Study of $CD4^+CD25^+$ T Cell-Mediated Suppression

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Abstract Autoimmune gastritis (AIG) is an experimental model that closely resembles human autoimmune gastritis, the underlying pathology of pernicious anemia. Pathogenic $CD4^+$ T cells are reactive to the parietal cell autoantigen, H/K ATPase, and are controlled by $CD4^+CD25^+$ T cells in an immunosuppressive cytokine-independent manner. Comparison of $CD4^+CD25^+$ T cell-mediated suppression in other autoimmune models shows inconsistencies with respect to requirements of cytokines for immunosuppression. More recent data, however, indicate that the evidence for requirement of IL-10 and TGF-β could be due to the complex nature of the T cells causing the disease as well as the role of induced regulatory T cell populations. AIG provides a well-defined model that may allow for better analysis of $CD4^+CD25^+$ T cell in vivo biology. Evidence from this model indicates that immune responses must be initiated and then $CD4^+CD25^+$ T cells are recruited to control the quality of the immune response.

Abbreviations

AIG	Autoimmune gastritis
IBD	Inflammatory bowel disease
d3Tx	Thymectomy on day 3 of life
Treg	Regulatory T cell
GITR	Glucocorticoid-induced TNF receptor
gLN	Gastric LN
DC	Dendritic cell

1 Introduction

It is now approaching 10 years since the identification of a naturally occurring $CD4^+$ T cell with constitutive expression of CD25 (IL-2Rα) as a potent suppressor T cell population capable of controlling immune pathology (Sakaguchi et al. 1995). Initial in vivo phenomena centered around their ability to suppress the induction of organ-specific autoimmune disease induced by day 3 thymectomy (d3Tx) of certain mouse strains (Asano et al. 1996; Suri-Payer et al. 1998) or by transfer of autoeffector T cells to immunocompromised animals (Sakaguchi et al. 1995). Today, these cells are recognized as major players in immune responses to not only autoantigens, but also allo, tumor- and bacteria/viral/parasitic-antigens (McHugh and Shevach 2002b).

Following in vitro characterization of $CD4^+CD25^+$ T cells from mice, it was confirmed that there was a homologous population of $CD4^+CD25^+$ T suppressor cells in humans with in vitro characteristics similar to those of mice (Shevach 2001). Recently, investigators have begun analysis of this cell population in various human disease states. Indeed, there has been a correlation between the frequency or functional reduction of $CD4^+CD25^+$ T cells and various autoimmune and allergic disorders (Kukreja et al. 2002; Ling et al. 2004; Salama et al. 2003; Viglietta et al. 2004).

Although first described by their control over immune responses in vivo, the greatest characterization of these cells has been carried out in vitro. Recent work is now dedicated to elucidating their activities in vivo, trying to translate their in vitro activities to in vivo therapy. Interestingly, some of the outcomes of in vivo biology of $CD4^+CD25^+$ T cells have not been predicted by their in vitro functions. As well, their in vivo activity in controlling various diseases has also shown some inconsistencies.

Since the discovery of the $CD4^+CD25^+$ T suppressor cell population, many different types of $CD4^+$ regulatory cells (Tr1, Th3), mainly induced in vitro, have been reported (Shevach 2002). Additionally, the number of immune

models used to define these cell types has grown exponentially. These in vivo models differ in requirements for induction of disease, cell types involved and the contribution of environmental and genetic factors. It has recently been put forth that some of the inconsistencies could lie with confusion between the involvement of naturally occurring Tregs and induced Tregs in these disease models (Piccirillo and Shevach 2004). As well, the various immune models may require different modes of suppressive activity, therefore leading to controversy over the $CD4^+CD25^+$ T cell mechanism of suppression.

The purpose of this review is twofold. It is becoming well established that $CD4^+CD25^+$ T cells play a role in a variety of immune responses. With so many variables that complicate an in vivo system, combined with the heterogeneity and complexity of a polyclonal $CD4^+CD25^+$ T cell population, it is difficult to control every parameter. Autoimmune gastritis (AIG) presents a well-characterized in vivo model for elucidation of $CD4^+CD25^+$ T cell in vivo biology. AIG has a known autoantigen, H/K ATPase, and strict requirement for disease induction by $CD4^+$ T cells and disease suppression by $CD4^+CD25^+$ T cells. H/K ATPase-specific TCR transgenic mice have been generated and are able to induce severe AIG, therefore eliminating the need for a polyclonal autoeffector population ($CD4^+CD45Rbhi$ or $CD4^+CD25^-$). Secondly, I would like to describe a model where $CD4^+CD25^+$ T cell-mediated suppression does not occur in the steady state, but requires appropriate immune activation of autoaggressive effectors to manifest.

2
Milestones in $CD4^+CD25^+$ In Vitro Characterization

Recently, $CD4^+CD25^+$ T cells have been expertly reviewed elsewhere summarizing their origin, in vitro activity and possible therapeutic potential (Sakaguchi 2004). I would like to highlight some of the landmarks of in vitro characterization and how these aspects of $CD4^+CD25^+$ biology may relate to the in vivo model of autoimmune suppression.

2.1
Development of an In Vitro Assay System of Suppression

With the demonstration that $CD4^+CD25^+$ T cells were capable of inhibiting the induction of autoimmune disease, several groups set out to purify this subset and develop an in vitro assay system to test the suppressive function of these cells. It was shown that soluble anti-CD3-induced $CD4^+$ T cell proliferation could be inhibited when increasing numbers of $CD4^+CD25^+$ T cells were added

(Takahashi et al. 1998; Thornton and Shevach 1998). Unlike $CD4^+CD25^-$ cells, the $CD25^+$ subset was unable to proliferate to TCR stimulation alone and required IL-2 or IL-4 for growth. These cells were subsequently classified as anergic; however, this term should be classified as their strict need for exogenous IL-2, as $CD4^+CD25^+$ T cells, as a population, are highly responsive to TCR stimulation (see Sect. 2.2).

The ability of $CD4^+CD25^+$ T cells to inhibit proliferation of the responding T cell population lay in their ability to inhibit the transcription of IL-2 in the responding population. Indeed, reagents such as IL-2 and agonistic anti-CD28 were able to restore the proliferative capacity of the responding T cells (Takahashi et al. 1998; Thornton and Shevach 1998). Some investigators hypothesized that the anergic state of the $CD4^+CD25^+$ subset was important for their suppressive activity, and breaking this state, by such reagents, would subsequently turn off their suppressive activity (Takahashi et al. 1998). Recently, Thornton et al. (2004b) have shown that stimulation of $CD4^+CD25^+$ T cells with IL-2 or IL-4 is crucial to arm these cells with their suppressive activity. In this study, both T cell proliferation and IL-2 mRNA levels were analyzed during the in vitro suppression assay. As expected, if anti-IL-2 was added during the suppressive co-culture, all proliferation was blocked, but $CD4^+CD25^+$ cells were unable to suppress IL-2 message in the responding T cells. This demonstrated that $CD4^+CD25^+$ T cells need both a TCR stimulus and IL-2 signaling to suppress the continued production of IL-2 by responding T cells.

This study (Thornton et al. 2004b) was interesting because it looked at a block in IL-2 message as a read-out of suppression rather than proliferation of the co-culture. As mentioned above, if IL-2 is added to the suppressive co-culture, proliferation is restored and therefore thought to overcome suppression. When IL-2 message was analyzed in the same co-culture, however, it was suppressed. This indicates, along with the above data, that addition of IL-2 does not overcome suppression, but is rather necessary for full suppressor function.

In vivo, mice deficient in IL-2, IL-2 signaling and co-stimulatory molecules, have low to no $CD4^+CD25^+$ T cells demonstrating a need for responsiveness to IL-2 for their generation (Sakaguchi 2004). It is also a possibility that low levels of IL-2, in addition to affecting their generation and maintenance, may also influence their suppressive abilities. Translating this data into an in vivo hypothesis, this could indicate that an immune response, inducing local production of IL-2 by responding cells must occur before $CD4^+CD25^+$ T cells can suppress the effector cells.

The in vitro assay system also demonstrated a requirement for TCR ligation in the induction of suppressive activity. It was shown that $CD4^+CD25^+$ T cells

needed to be activated through their TCR and the resulting suppression was cell-contact-dependent (Thornton and Shevach 1998). Once activated, this suppressive activity no longer depended on antigen and could suppress any T cell nonspecifically (Thornton and Shevach 2000). Additionally, there was no requirement for the same MHC:peptide complexes presented on the same APC for suppression to occur. These studies indicate the potential for any immune response to be suppressed as long as $CD4^+CD25^+$ T cells receive a TCR and IL-2R stimulus. This preactivation also increased the suppressive potency of the cells by up to fourfold. With such potent nonspecific suppression following activation of $CD4^+CD25^+$ T cells, it is difficult to imagine that any immune response would be induced. Therefore regulation of $CD4^+CD25^+$ T cell activity and its timing during an immune response is critical.

2.2 Genetic Analysis of $CD4^+CD25^+$ T Cells

This activation requirement for suppressive activity led to the hypothesis that molecules, most likely cell surface, needed to be induced and once expressed would allow for nonspecific suppression. Several investigators began analysis of genes specifically upregulated by $CD4^+CD25^+$ subsets (Gavin et al. 2002; McHugh et al. 2002; Zelenika et al. 2002). $CD4^+CD25^+$ T cells were highly responsive to this stimulation, upregulating approximately four times as many genes as their $CD25^-$ counterparts. This analysis tried to answer several questions.

1. What is the suppressive molecule?
2. How do $CD4^+CD25^+$ cells maintain their anergic state?
3. Can $CD4^+CD25^+$ T cells be further subdivided into suppressive and nonsuppressive subsets?

Although none of these questions were fully answered, a more detailed phenotype of these cells was delineated. Since this analysis, even more molecules (Bruder et al. 2004), such as the transcription factor FoxP3 (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003), have been identified as markers for $CD4^+CD25^+$ T cells. By identifying more cell surface molecules on $CD4^+CD25^+$ T cells, certain subsets, mainly based on bimodal expression of CD62L (Szanya et al. 2002) and CD103 (Lehmann et al. 2002), and recently other integrins (Stassen et al. 2004), were shown to have distinct suppressive activity in vivo or altered function in vitro. Although CD25 still remains the most reliable marker in terms of suppressive activity in vitro, subsets expressing CD103 or CD62L have been shown to more efficiently suppress colitis or diabetes, respectively. It is interesting to note that $CD103^+$ $CD4^+CD25^+$ T cells

have a greater number of $CD62L^{lo}$ cells (R.S.M., unpublished observation). Could this indicate that in different tissues and with different diseases, subsets within the $CD4^+CD25^+$ T cell population can display different functions?

Analysis of freshly isolated $CD4^+CD25^+$ T cells revealed the constitutive expression of the glucocorticoid-induced TNF receptor (GITR), a member of the TNFR superfamily (McHugh et al. 2002; Shimizu et al. 2002). Two groups independently determined that antibodies to this molecule were able to restore proliferation of the responding cells in an in vitro suppression assay. This receptor was not found to be the suppressor effector molecule, but engagement of GITR on $CD4^+CD25^+$ T cells was thought to downregulate their ability to suppress. Although anti-GITR negated the suppressive activity of $CD4^+CD25^+$ T cells, its stimulation of $CD4^+CD25^+$ T cells increased their responsiveness to IL-2 (McHugh et al. 2002).

Several groups have now cloned the ligand for GITR and have shown that engagement of GITR with its natural ligand will also overcome suppressive activity of $CD4^+CD25^+$ T cells (Kim et al. 2003; Stephens et al. 2004; Tone et al. 2003). Many of these reports demonstrate that GITR engagement on $CD4^+CD25^-$ T cells is co-stimulatory and one study actually demonstrates, utilizing $GITR^{-/-}$ mice, that engagement of GITR on the responding T cells, but not $CD4^+CD25^+$ T cells as previously hypothesized, is required for reversal of suppression (Stephens et al. 2004).

Consistently, interactions of GITR with its ligand are shown to overcome the ability of $CD4^+CD25^+$ mediated suppression in vitro and in some instances in vivo (Kohm et al. 2004; Shimizu et al. 2002); therefore it is necessary to determine where, when and how GITR-L is expressed during an immune response. Several groups have shown that GITR-L is constitutively expressed on various APCs and this expression is down-regulated with activation signals, such as engagement of TLRs in vitro (Stephens et al. 2004; Tone et al. 2003). This timing of expression is important, indicating that at the initial stages of APC activation and T cell priming, GITR is possibly engaging GITR-L, and therefore $CD4^+CD25^+$ T cells are unable to suppress (Fig. 1). But with further maturation of APCs, GITR-L is down-regulated, no longer having a hold on suppression. While responding T cells are being stimulated by APCs, $CD4^+CD25^+$ T cells can expand by stimulation through their TCR, GITR and local IL-2 production. Once GITR-L is down-regulated, $CD4^+CD25^+$ T cells are at sufficient numbers to begin quelling the immune response. The nature of the T cell response, whether self-antigen or foreign-antigen, may additionally influence how easily $CD4^+CD25^+$ T cells can suppress a response. A careful analysis of the pattern and timing of GITR-L expression in vivo during various immune responses is necessary to determine the possible roles these interactions play in activation of T cell responses as well as their suppression.

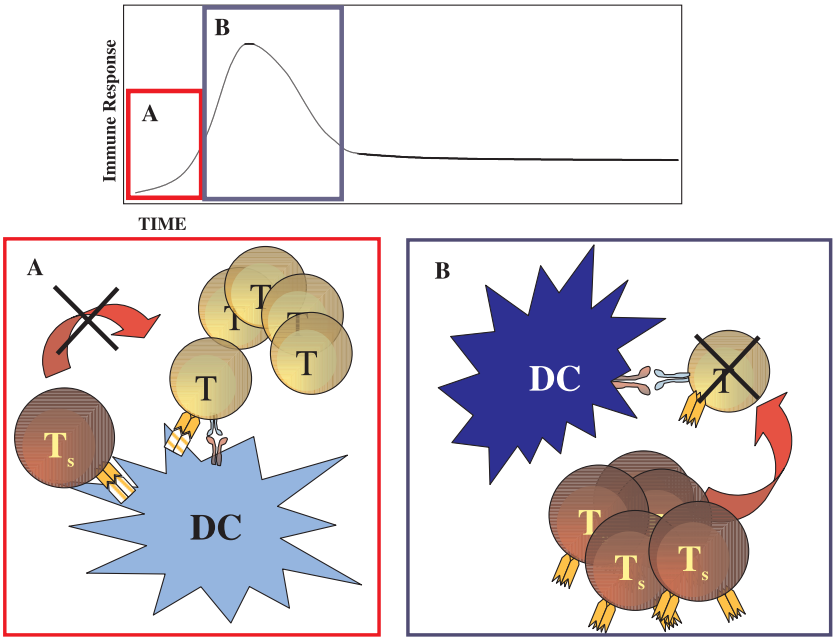

Fig. 1A, B Model of GITR/GITR-L interactions for the control of immunosuppression. At the initiation of the immune response (**A**), DCs are able to present antigen to effector T cells (T_e). Interactions with GITR-L enable Te to overcome regulation by T_s and therefore have an advantage in activation. At this same time GITR-L stimulation of $CD4^+CD25^+$ T cells allow for increased sensitivity to IL-2 and proliferation. As the immune response progresses, GITR-L is downregulated on the DCs, allowing $CD4^+CD25^+$ T cells to control activation of naïve T_e as well as effector T_e. As $CD4^+CD25^+$ T cells have expanded, they are in sufficient numbers to control the immune response

3 Autoimmune Gastritis Model of $CD4^+CD25^+$ T Cell-Mediated Suppression

Over the years, many autoimmune models have been employed to investigate the in vivo biology of suppressor T cells (Shevach 2000). One of the original autoimmune models used in the study of $CD4^+CD25^+$ biology is AIG induced by thymectomy on day 3 of life (d3Tx) or $CD25^-$ T cell transfer to immunocompromised animals. Another widely utilized autoimmune disease model is inflammatory bowel disease (IBD) or colitis. Transfer of $CD4^+CD45Rb^{hi}$ (Powrie et al. 1994) or $CD4^+CD25^-$ T cells (Liu et al. 2003;

Suri-Payer and Cantor 2001) into SCID or $RAG^{-/-}$ mice will induce this wasting disease. This model is comprehensively reviewed in this issue, but will be discussed in comparison with AIG as two model systems used to elucidate $CD4^+CD25^+$ T cell suppressive activity in vivo.

3.1 Immunopathology of AIG

Experimental AIG in mice resembles human autoimmune gastritis, the underlying pathology of pernicious anemia (Alderuccio et al. 2002). Pernicious anemia is one of the more prevalent autoimmune diseases and is the most common cause of vitamin B_{12} deficiency, as antibodies and T cells target cells that produce intrinsic factor (Toh et al. 1997). Gastritis in humans and mice is characterized by mononuclear cell infiltrates within the gastric mucosa and submucosa and production of autoantibodies reactive against gastric parietal cells.

Circulating autoantibodies are closely associated with disease pathology in both humans and mice, with the majority of the antibodies reactive against the parietal cell proton pump, H/K ATPase (Jones et al. 1991). Autoreactive T cell clones identified in humans (Bergman et al. 2003; D'Elios et al. 2001) and mice (De Silva et al. 1999; Katakai et al. 1997; Suri-Payer et al. 1999) have been found specific for H/K ATPase as well, and common peptide epitopes are shared between the species (Bergman et al. 2003).

Severe AIG results in loss of parietal and chief cells. This destruction of the gastric mucosa cellular architecture has been suggested to be FasL-dependent (Marshall et al. 2002; Nishio et al. 1996). A role for IFN-γ(Barrett et al. 1996), but not TNF-α(Marshall et al. 2004), in the initiation of disease has been demonstrated as anti-IFN-γ antibody treatment early in disease induction blocks AIG. Loss of parietal cell production of intrinsic factor results in vitamin B_{12} deficiency leading to gastric, blood and neurological disorders (Toh et al. 1997).

3.2 The H/K ATPase Autoantigen

The cellular and humoral immune response has been seen to both chains of the H/K ATPase heterodimer (Toh et al. 2000), but the majority of reported $CD4^+$ T cells are seen to be reactive to alpha chain peptides. There is still controversy over which chain, if any, of H/K ATPase is important in the initiation of AIG. Studies using mice transgenic for α (H/Kα:I-E) or β (H/Kβ:I-E) chain under the control of a class II promoter targeting expression to the thymus

indicates the β chain is the critical autoantigen target (Alderuccio et al. 1997). The d3Tx of H/Kβ:I-E mice did not result in AIG, suggesting that β chain expression in the thymus negatively selects autoreactive T cells. Interestingly, α chain message is naturally expressed in the thymus during gestation and after birth, but is insufficient for complete deletion of all α chain reactive TCRs (Alderuccio et al. 1997). Actually, H/K ATPase reactive TCR transgenic thymocytes from A23 mice (see Sect. 3.3) are well selected and skewed toward CD4 single positive cells (McHugh et al. 2001b).

An alternate explanation as why *H/Kβ*: I-E transgenic mice do not develop AIG after d3Tx is that β chain expression is required for full protein expression and assembly of both chains (Gottardi and Caplan 1993). Therefore β chain rescues α chain expression, leading to subsequent deletion of α and β reactive T cells. This possibility is actively being investigated.

3.3 Pathogenic CD4$^+$ T Cells

Several groups have demonstrated that CD4$^+$ T cells are the pathogenic cells in AIG (Alderuccio et al. 2002). Autoantibodies are insufficient to transfer disease and there have been no requirements for B cells or CD8$^+$ T cells for induction of disease. Within the chronic lesions of AIG, T cells, B cells and APCs associate in an organized tertiary lymphoid structure possibly supported by CXCL13, CCL4, CCL5, CXCL9 and CXCL10 production (Katakai et al. 2003). Although the expression of these chemokines may indicate a Th1-biased environment, many cytokines associated with a Th2 profile have been detected in the gastric mucosa of AIG$^+$ mice (Martinelli et al. 1996). These include IL-5 and IL-10; however, there is a notable absence of IL-4 production. Indeed, both Th1- and Th2-skewed CD4$^+$ gastritic clones have been isolated from the gastric LN of AIG$^+$ mice (Suri-Payer et al. 1999).

Several H/K ATPase reactive T cell clones have been characterized. Two clones reactive to the α chain isolated from AIG$^+$ animals after d3Tx have been described (Suri-Payer et al. 1999). TxA23 (α630–641) has a typical Th1 profile secreting IFNγ and TNFα. Upon transfer to immunocompromised mice, they induce severe AIG with the characteristic mononuclear cell infiltrate. TxA51 (α889–900) displays a Th2 pattern of differentiation, producing IL-4, IL-10 (Suri-Payer et al. 1999) and IL-5 (S. Chegini and E. Shevach, unpublished observations). These cells also induced a severe pathology upon transfer to immunocompromised mice; however, infiltrate into the gastric mucosa primarily consisted of eosinophils. Although the polyclonal lesion seems to be significantly Th1-influenced, Th2 cells are capable of differentiation and causing disease pathology. Both these clones are controlled with co-transfer of

$CD4^+CD25^+$ T cells, demonstrating suppression of both naïve ($CD4^+CD25^-$) and effector T cells (gastritic clones).

A23 (McHugh et al. 2001b) and A51 (Candon et al. 2004) transgenic mice have been developed from the cloned TCRs of TxA23 (Vα2.6/Vβ2) and TxA51 (Vα17.3/Vβ4), respectively. A23 mice display 100% penetrance of disease on susceptible backgrounds. The T cells isolated from A23 mice display a Th1 cytokine profile and show strong activation within the draining gastric lymph node (gLN). Signs of disease and activation are seen as early as day 10 of life. These animals are capable of generating $CD4^+CD25^+$ T cells that can be activated by the H/K ATPase self-antigen; however, these cells are not capable of controlling the immune pathology in the transgenic mice. This inability of $CD4^+CD25^+$ suppressor activity could be secondary to the overall increase in autoreactive T cell precursors. Other TCR transgenics reactive to self-antigens, especially those specific for myelin proteins (Lafaille et al. 1994), however, do seem to be controlled by regulatory T cells. The disease incidence in these mice remains low and usually requires immunization with myelin proteins or crossing to a $RAG^{-/-}$ background to induce disease.

AIG has been described in mice carrying a single TCR α chain transgene (Sakaguchi et al. 1994). These mice spontaneously develop AIG, but also have $CD4^+CD25^+$ cells present in the periphery of an adult (A. Thornton, unpublished observation). One hypothesis is that introduction of atypical gene expression within the thymus could delay the development of $CD4^+CD25^+$ cells, which generally begin to emigrate on day 3–4 of life. This delay, in combination with neonatal lymphopenia and early, localized expression of autoantigen, could result in autoimmune disease. All these circumstances, as well as increased autoreactive T cells, could lead to this fulminant disease.

A51 mice also displayed signs of AIG, however at a lower incidence, 50%–80%, and it is manifested later in life, around 10 weeks (Candon et al. 2004). TCR transgenic T cells isolated from the gLN were primed to produce Th2 cytokines. Similar to transfer of TxA51 clone, the A51 mice had a polymorphonuclear cell infiltrate composed of mostly eosinophils. Interestingly, the H/K ATPase peptide that A51 TCR transgenic T cells are reactive against is not efficiently presented by APC in vitro. This may account for the lower incidence and delay in disease onset.

A third TCR transgenic mouse with T cells reactive to H/K ATPase has been described by Alderuccio et al. (2000). The TCR was cloned from a T cell hybridoma generated from AIG^+ mice immunized with H/K ATPase β $chain_{253–277}$ peptide. However, disease only developed spontaneously in about 20% of the mice. The T cells in these mice were not selected well in the thymus, perhaps indicating the TCR affinity for this peptide is not efficient for positive selection. Furthermore, the low incidence of spontaneous disease

could be because these autoreactive T cells were only revealed following immunization of peptide in CFA. Therefore it is possible that this form of stimulation is required for their disease-inducing potential.

3.4 Dendritic Cells Presenting H/K ATPase

How autoreactive $CD4^+$ T cells become activated to initiate autoimmune disease is an actively investigated area of the pathophysiology of many autoimmune diseases. In the peripheral tissues, dendritic cells (DCs) sample the environment, acquiring self-antigens from tissue sites, perhaps by the phagocytosis of apoptotic cells (Steinman et al. 2003). These DCs migrate to the draining lymph nodes where they present the antigen in the context of MHC to specific T cells. It has recently been shown that the autoantigen H/K ATPase is processed and presented in the gastric LN of unmanipulated BALB/c mice (Scheinecker et al. 2002). DCs likely pick up this antigen in the gastric mucosa, ingesting naturally apoptotic parietal cells. In this regard, close contact between DCs and parietal cells in the gastric mucosa has been observed.

Purifying $CD8\alpha^+$ and $CD8\alpha^{lo/-}$ DCs from gLN demonstrated direct presentation of an in situ processed autoantigen to H/K ATPase reactive clones. This was confirmed in vivo with proliferation of A23 T cells exclusively within the gLN of unmanipulated BALB/c mice (Scheinecker et al. 2002). A23 T cells adoptively transferred into BALB/c mice, like other self-reactive T cells, expanded and then subsequently contracted and could not be detected 3 weeks after adoptive transfer (R. DiPaolo and E. Shevach, unpublished observation).

Although there is constant turnover and presentation of the autoantigen in the steady state, this does not lead to spontaneous autoimmunity. There are several possibilities to explain why there is not a progression to autoaggression. First, the presence of $CD4^+CD25^+$ T cells could continually suppress DC activation of autoreactive T cells. Depletion of $CD25^+$ cells, however, with anti-CD25 antibody, rarely leads to the induction of autoimmunity (McHugh and Shevach 2002a). This indicates that another level of peripheral tolerance is possibly involved. The DCs may present H/K ATPase in a tolerogenic manner, as previously reported with the presentation of other self-antigens within the draining LN (Steinman et al. 2000, 2003). Therefore, $CD4^+CD25^+$ T cells may not be necessary to control presentation of self-antigen in this context. For autoimmunity to manifest, autoreactive T cells would need to be activated with mature DCs capable of immune stimulation. AIG can be induced after H/K ATPase immunization (Scarff et al. 1997) or localized transgenic-production of GM-CSF (Biondo et al. 2001).

3.5
Models of AIG Induction

AIG can be induced by various means. These models have been separated into four main categories: (1) lymphopenic, (2) nonlymphopenic, (3) transgenic, and (4) spontaneous (Alderuccio et al. 2002). The most common model is thymectomy on day 3 of life (d3Tx). In susceptible strains of mice, such as BALB/c, d3Tx resulted in AIG as well as several other organ-specific autoimmune diseases (Kojima and Prehn 1981). The underlying cause of this was the early removal of $CD25^+$ suppressor cells in combination with a state of lymphopenia. The role of $CD25^+$ cells was confirmed when Sakaguchi et al. could reproduce the same autoimmune profile in immunocompromised mice upon transfer of $CD25^-$ T cells (Sakaguchi et al. 1995).

The autoimmune diseases induced by both methods, as well as by gastritis-inducing clones, could be completely suppressed with co-transfer of $CD25^+$ T cells if given within 1 week of induction of disease (McHugh et al. 2001a). The suppression was less effective if $CD25^+$ T cells were transferred after that time point, indicating that $CD25^+$ T cell suppression may not be efficient once a certain level of T cell activation or pathology has begun. This contrasts $CD25^+$ T cell control of IBD. Pathology in the colon would eventually subside if $CD4^+CD25^+$ T cells were given 4 weeks after disease induction (Mottet et al. 2003); however, transfer of Tr1 clones were able to immediately halt disease progression (Foussat et al. 2003). It is interesting that in both AIG and

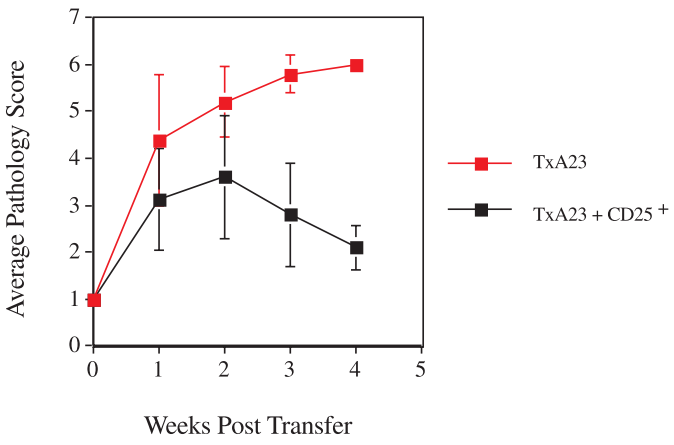

Fig. 2 Autoimmune pathology is initiated in the presence of $CD4^+CD25^+$ T cells. TxA23, an H/K ATPase-specific clone, was co-injected with $CD4^+CD25^+$ T cells into BALB/$c^{nu/nu}$ mice. Every week after adoptive transfer, stomachs were harvested, H&E stained and scored for AIG pathology

IBD, $CD4^+CD25^+$ T cell suppression is not immediate. Analysis of the gastric mucosa at early time points after adoptive transfer of effector cells revealed a transient lymphocytic infiltrate into the tissue even in the presence of suppressor cells (Fig. 2). $CD4^+CD25^+$ T cells, if co-transferred would eventually suppress AIG and infiltration would clear by 4 weeks.

4
$CD4^+CD25^+$ T Cell-Mediated Suppression of AIG

4.1
Involvement of Lymphopenia

Although not categorized as lymphopenic, all the models known to induce AIG involve a certain degree of lymphopenia, either due to thymectomy, the use of T cell-deficient animals or the neonatal period of life (Min et al. 2004). An initial hypothesis as to how $CD25^+$ T cells were controlling induction of disease was by inhibiting or limiting the homeostatic/lymphopenia-induced proliferation of the effector cells. There have been data for and against $CD4^+CD25^+$ T cell control of lymphopenia-induced proliferation. Annacker et al. (2001) demonstrated that co-transfer of $CD4^+CD25^+$ T cells would decrease the early proliferation of $CFSE^+CD4^+CD25^-$ T cells and could control peripheral T cell numbers at later time points. Other groups (Martin et al. 2004; McHugh and Shevach 2002a) have failed to see this same effect on the early proliferation of effector cells, and the modest inhibition seen by Annacker et al. (2001) might be due to an overall increase in T cells transferred. As previously mentioned, in immunodeficient animals receiving $CD25^-$ and $CD4^+CD25^+$ T cells, the overall accumulation of T cells in the periphery is significantly reduced compared to $CD4^+CD25^-$ T cell transfer alone. This must be interpreted with caution, however, as immunodeficient mice receiving $CD4^+CD25^-$ cells alone develop severe immunopathology, which can lead to a greater expansion of peripheral T cells.

Suppression of IBD has recently been shown to be controlled by not only known suppressor cell populations ($CD4^+CD25^+$ and $CD4^+CD45Rb^{lo}$), but by T cells in the naïve cell pool (Barthlott et al. 2003). By transfer of increasing numbers of $CD4^+CD45Rb^{hi}$ cells, or even monoclonal TCR transgenic T cells, to immunocompromised mice, the incidence of IBD declined. This regulatory activity was associated with high proliferation potential upon transfer, indicating that rapid expansion, and therefore, filling of the empty space would lead to control of immunopathology. In contrast to this model, transfer of high numbers of $CD4^+CD25^-$ T cells did not inhibit the induction of AIG

(R.S.M., unpublished observations), again indicating that different disease models may be controlled by different mechanisms of suppression.

To attempt to separate depletion of $CD25^+$ T cells and lymphopenia, McHugh and Shevach (2002a) set out to induce AIG by antibody depletion of $CD25^+$ cells in vivo. Contrary to previous data (Taguchi and Takahashi 1996), AIG was rarely induced by $CD25^+$ T cell depletion. This observation has been confirmed by several groups looking to induce autoimmune disease (Laurie et al. 2002) or tumor immunity (Onizuka et al. 1999; Shimizu et al. 1999; Sutmuller et al. 2001). The depletion in vivo was effective as splenocytes from these mice were able to transfer AIG to immunocompromised animals (McHugh and Shevach 2002a). $CD25^+$ cells do eventually reconstitute the animals, but in our hands $CD25^+$ T cell levels were still depleted 6 weeks after antibody treatment. It is possible that AIG is controlled by the reconstituting $CD25^+$ cells, but it is also possible that in the steady state there are other tolerance-inducing mechanisms that control autoaggressive T cells.

Although $CD25^+$ cell depletion alone was insufficient to induce AIG, depletion in combination with immunization of H/K ATPase in IFA induced chronic severe AIG (McHugh and Shevach 2002a). H/K ATPase immunization in CFA will induce a limited amount of pathology; however, once the immunization is ceased, pathology will recede (Scarff et al. 1997). In total, these observations lead to a model where AIG requires both depletion of $CD25^+$ T cells and a strong stimulus for autoreactive cells, such as immunization, inflammation of the tissue, or lymphopenia (Fig. 3). $CD4^+CD25^+$ T cells play a role in control of autoimmune activation in this context, but in the steady state, other tolerogenic mechanisms, such as immature DCs may control autoeffector cells (Steinman et al. 2003).

4.2 Involvement of Immunosuppressive Cytokines

Within the biology of $CD4^+CD25^+$ T cells, there has yet to be anything more controversial than the role of immunosuppressive cytokines. The in vitro suppression assay system allowed for a model of $CD4^+CD25^+$ T cells that mediated suppression in an activation-dependent, contact-dependent, and cytokine-independent manner (Thornton and Shevach 1998). Most researchers investigating $CD4^+CD25^+$ cells in the mouse and human system have not been able to overcome suppression by adding antibodies to immunoregulatory cytokines IL-4, IL-10 or TGFβ (Shevach 2002). Additionally, $CD4^+CD25^+$ cells from IL-4-, IL-10- (Thornton and Shevach 2000) or TGFβ- (Piccirillo et al. 2002) deficient animals were as equally suppressive as wild-type $CD4^+CD25^+$ T cells in vitro. In vivo data from IBD (Asseman et al. 1999; Powrie et al. 1996)

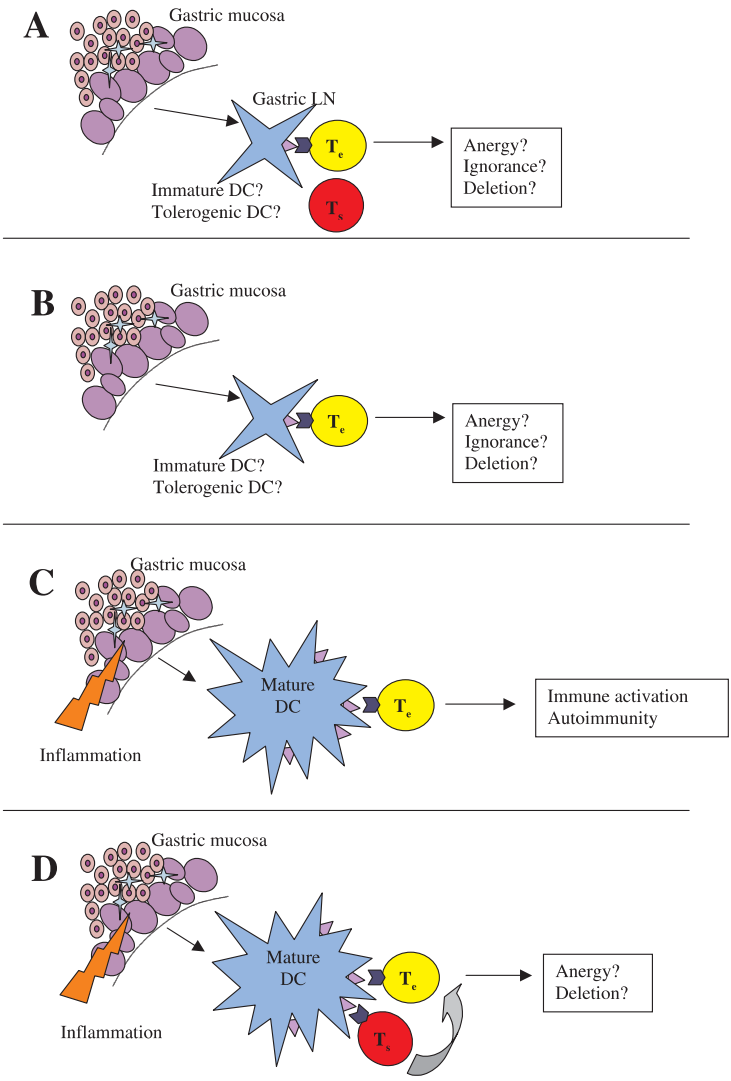

Fig. 3A–D In the absence of $CD4^+CD25^+$ T cells, additional signals are needed to initiate autoimmunity. In the steady state (**A**), autoreactive effector cells are kept in check by multiple mechanisms, $CD4^+CD25^+$ T cells or tolerogenic DCs. Depletion of $CD4^+CD25^+$ T cells does not lead to autoimmunity, indicating another mechanism for control of autoreactive T cells (**B**). Depletion of $CD4^+CD25^+$ T cells in combination with tissue inflammation, or immunization, leads to autoimmunity that is not controlled by other tolerance mechanisms (**C**). In the presence of $CD4^+CD25^+$ T cells, inflammation may activate the autoreactive T cells, but there autoactivity is kept in check by $CD4^+CD25^+$ T cells (**D**)

and other models (Cameron et al. 1997; Homann et al. 1999; Krause et al. 2000; Seddon and Mason 1999) have indicated a role for IL-4, IL-10 and/or TGFβ in suppression of autoimmune disease. Antibodies blocking either IL-10 or TGF-β were able to reverse suppression mediated by $CD4^+CD45Rb^{lo}$ cells. As well, IL-$10^{-/-}$ mice on susceptible backgrounds developed IBD. Interestingly, these same animals did not develop gastritis (Suri-Payer and Cantor 2001).

When Nakamura et al. (2001) published their study identifying membrane-bound TGF-β on the surface of resting and activated $CD4^+CD25^+$ T cells, this seemed to be a link between the necessity for cell contact and the dependency of TGF-β in in vivo model systems. Although TGF-β may play a role in $CD4^+CD25^+$ T cell-mediated suppression or activation, it is not essential for their activity in vitro (Piccirillo et al. 2002). It has been demonstrated that $CD4^+CD25^+$ T cells purified from the thymus of TGF-β-deficient mice are fully functional suppressors. Moreover, several genetically modified responder T cells incapable of inhibition by TGF-β are equally suppressible by $CD4^+CD25^+$ T cells.

To address whether immunosuppressive cytokines played a role in AIG, splenocytes from IL-4- or IL-10-deficient animals were used as a source of suppressor cells in the d3Tx or CD25- T cell transfer model. IL-4 and IL-$10^{-/-}$ $CD4^+CD25^+$ were as efficient as wild-type BALB/c in suppression of AIG (McHugh et al. 2001a; Suri-Payer and Cantor 2001). This indicates that IL-4 or IL-10 production by $CD4^+CD25^+$ T cells is not necessary for the inhibition of AIG. This lack of a requirement for IL-10 in AIG suppression is in contrast to previous studies with IBD. Recently, however, long-lived autoreactive memory cells have been identified within the $CD4^+CD45Rb^{lo}$ or $CD4^+CD25^+$ suppressor cell pool, especially if purified from the mesenteric LN (Asseman et al. 2003). Adoptive transfer of these suppressor populations in combination with anti-IL-10R antibody revealed pathogenic cells capable of inducing IBD. In the same study, it was also reported that splenic $CD4^+CD25^+$ T cells purified from IL-10-deficient animals were capable of suppressing naïve $CD45Rb^{hi}$ cells. Therefore it seems that control of memory effectors ($CD45Rb^{lo}$) is IL-10-dependent, but suppression of naïve cells ($CD45Rb^{hi}$) is less dependent on IL-10.

$CD4^+CD25^+$ T cells are capable of suppressing AIG induced by naïve ($CD25^-$) and effector (clones) T cells (Suri-Payer et al. 1998). It has not been determined, however, whether IL-10 production is required for inhibition of AIG induced by the fully differentiated gastritic clones. It is possible that IL-10 is necessary for suppression of these autoaggressive effectors. Unlike IBD, it has not been demonstrated that within the $CD4^+CD25^-$ pool of cells resides previously activated H/K ATPase reactive memory T cells. It is actively being investigated whether potential H/K ATPase reactive T cells are aner-

gized, deleted or just kept in check by $CD4^+CD25^+$ T cells. A recent report utilizing the IBD model indicates that $CD4^+$CD45Rbhi cells that have been suppressed by co-transfer of $CD4^+CD25^+$ T cells are still capable of inducing IBD if transferred alone to another immunodeficient mouse (Martin et al. 2004).

Use of anti-IL-10R indicates that perhaps cell types, other than $CD4^+CD25^+$ cells, produce IL-10 necessary for suppression of immunopathology. In vitro work using human $CD4^+CD25^+$ T cells has identified their potential for infectious tolerance, instructing $CD25^-$ T cells to make IL-10 (Dieckmann et al. 2002) or TGF-β (Jonuleit et al. 2002). Moreover, in vivo work by Foussat et al. (2003) has hinted at such a mechanism where $CD4^+CD25^+$ T cells may play a role inducing other cells to produce IL-10.

TGF-β is another candidate immunosuppressive cytokine that has been implicated in control of autoimmune disease. In the $CD25^-$ T cell adoptive transfer model of AIG, we employed anti-TGF-β antibodies with co-transfer of $CD4^+CD25^+$ cells and saw no decrease in efficiency of suppression (Piccirillo et al. 2002). Again, this is a difference between suppression requirements of AIG and IBD. Experiments using anti-TGF-β antibodies, however, cannot distinguish between $CD4^+CD25^+$ production of TGF-β and other cells' production. Therefore, one conclusion of this data is that $CD25^+$ T cells can instruct another cell, perhaps an induced Treg to produce TGF-β that can subsequently have an effect. Indeed this may be the case as recent data has shown that $CD4^+CD25^+$ T cells isolated from TGF-$\beta^{-/-}$ mice are able to suppress IBD, but in a TGF-β dependent manner (F. Powrie, personal communication).

4.3 Involvement of Co-stimulatory Molecules

$CD4^+CD25^+$ T cells constitutively express CTLA-4 (Read et al. 2000; Takahashi et al. 2000), the B7 counter-receptor responsible for downregulation of T cell responses (Walunas et al. 1994). CTLA-4-deficient mice suffer from massive lymphoproliferation and autoimmune pathology, leading to death within at a few weeks of life (Waterhouse et al. 1995). This defect in CTLA-4 was found to not be cell autonomous as a mixture of CTLA-$4^{+/+}$ and CTLA-$4^{-/-}$ bone marrow was able to control the immunopathology normally seen in a CTLA-4-deficient mouse (Bachmann et al. 1999). It was additionally demonstrated that in vitro and in vivo treatment with a blocking anti-CTLA-4 antibody would result in a lack of $CD4^+CD25^+$ T cell-mediated suppression (Read et al. 2000; Takahashi et al. 2000).

The role of CTLA-4 in AIG was addressed by anti-CTLA-4 treatment of immunocompromised mice that had received $CD4^+CD25^-$ cells alone or

with $CD4^+CD25^+$ T cells. These experiments did not reveal any effect on $CD4^+CD25^+$ T cells ability to inhibit AIG (McHugh et al. 2001a). Again, this suggests that individual models of disease have different requirements for suppression. In vitro analysis of the role of CTLA-4 has revealed that activation of the responding cell can influence the requirements for CTLA-4 signaling in $CD4^+CD25^+$ T cell-mediated suppression (Thornton et al. 2004a). Perhaps pathogenic T cells involved in IBD receive greater stimulation than those inducing AIG, and therefore the suppression is more reliant on CTLA-4 signals.

As mentioned in Sect. 2.1, mice deficient in IL-2, IL-2R and co-stimulatory molecules, such as CD28, have few to no $CD4^+CD25^+$ T cells. CD28-deficient mice have about 40%–50% of the $CD25^+$ cells found in wild-type BALB/c (McHugh et al. 2001a; Salomon et al. 2000). Although, $CD4^+CD25^+$ T cells from $CD28^{-/-}$ mice display suppressive activity, these cells were less efficient in suppressing AIG (McHugh et al. 2001a) and IBD (de Jong et al. 2004). It is not clear what the in vivo deficiency of these cells is, but may possibly be secondary to survival. Another possibility is the requirement $CD4^+CD25^+$ cells have for IL-2 signaling to support full suppressor functions.

It has recently been noted that GITR expression is not upregulated in the absence of CD28 signaling (Stephens et al. 2004). As previously demonstrated, GITR signaling increases IL-2 responsiveness (McHugh et al. 2002). Perhaps in $CD28^{-/-}$ mice, GITR levels are low, which lowers IL-2 responsiveness in vivo. Therefore, $CD28^{-/-}$ $CD4^+CD25^+$ T cells do not receive sufficient IL-2 stimulation for full suppressive activity.

5 Concluding Remarks

Throughout this review, several lines of in vitro and in vivo evidence have been compiled indicating the timing of $CD4^+CD25^+$ T cell suppression. First, $CD4^+CD25^+$ T cells require TCR and IL-2 stimulation for suppression. Second, lymphocytic infiltrate transiently occurs in the presence of $CD4^+CD25^+$ cells and may take up to 4 weeks to clear. Next, absence of $CD4^+CD25^+$ T cells does not alter tolerance to self-antigens in the steady state, but are essential following strong autoreactive stimulation. Lastly, GITR-L, whose engagement of GITR can prevent $CD4^+CD25^+$ T cell suppression of responding T cells, is expressed constitutively by APCs and is only down-regulated with APC maturation by TLR and other stimulations. Taken together, this suggests that $CD4^+CD25^+$ T cell suppression of self-reactivity does not come into play until DCs have had appropriate maturation to present self-antigen in an immunostimulatory manner. Recent analysis of in vivo immune responses

to immature or mature DCs demonstrates that the presence of $CD4^+CD25^+$ T cells only controls responses to mature DCs (Oldenhove et al. 2003). In the context of presentation of self-antigen by immature DCs, other tolerogenic mechanisms or other regulatory cells may play a role. As well, GITR-L could be expressed by immature DCs and therefore negate any suppressive mechanisms of $CD4^+CD25^+$ T cells. Such strong stimulatory signals from inflammation or lymphopenia would initial autoimmunity and subsequently its control.

With their therapeutic potential, it is becoming increasingly important to work out the suppressive mechanism of $CD4^+CD25^+$ T cells in vivo. The various cell types involved and requirements for disease induction, however, can complicate analysis in vivo. Indeed, the nature of the immune response or the state of T cell activation may require various means of control. The autoimmune disease AIG presents a well-defined in vivo model for the analysis of $CD4^+CD25^+$ T cell biology. A monoclonal $CD4^+$ T cell reactive to the known self-antigen, H/K ATPase, is solely capable of disease induction. Additional tools such as H/K ATPase transgenics and knockouts allow for analysis of the role of antigen-specificity of autoimmune suppression. In being able to control the initiating effector population as well as being able to visualize the DCs presenting the self-antigen, AIG provides an excellent tool for elucidating the in vivo mechanisms of $CD4^+CD25^+$ T cell suppression.

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