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Autoimmune Ovarian Disease in Day 3-Thymectomized Mice: The Neonatal Time Window, Antigen Specificity of Disease Suppression, and Genetic Control

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Abstract Discovery of the $CD4^+CD25^+$ T cells has stemmed from investigation of the AOD in the d3tx mice. Besides $CD4^+CD25^+$ T cell depletion, d3tx disease induction requires effector T cell activation prompted by lymphopenia. This is supported by other neonatal AOD models in which T cell-mediated injury has been found to be triggered by immune complex or Ag immunization. In addition, there is growing evidence that support a state of neonatal propensity to autoimmunity, which depends on concomitant endogenous antigenic stimulation, concomitant nematode infection, resistance to $CD4^+CD25^+$ T cell regulation, and participation of the neonatal innate system. The suppression of d3tx disease by polyclonal $CD4^+CD25^+$ T cells appears to be dependent on endogenous Ag and the persistence of regulatory T cells. Thus, suppression of AOD occurs in the ovarian LN, and AOD emerges upon ablation of the input regulatory T cells; and in AIP, the hormone-induced expression of prostate Ag in the $CD4^+CD25^+$ T cell donors rapidly enhances the capacity to suppress disease over Ag negative donors. Finally, genetic analysis of AOD and its component phenotypes has uncovered seven *Aod* loci. As the general themes that emerged, significant epistatic interactions among the loci play a role in controlling disease susceptibility, the majority of the *Aod* loci are linked to susceptibility loci of other autoimmune diseases, and the genetic intervals encompass candidate genes that are differentially expressed between $CD4^+CD25^+$ T cells and other T cells. The candidate genes include *Pdcd1,* TNFR superfamily genes, *H2, Il2, Tgfb, Nalp5* or *Mater*, an oocyte autoAg that reacts with autoantibody in sera of d3tx mice.

Abbreviations

Ab	Antibody
Ag	Antigen
AIG	Autoimmune gastritis
AOA	Antiovarian autoantibody
AOD	Autoimmune ovarian disease
AIP	Autoimmune prostatitis
APC	Antigen-presenting cell
AGM1	Asialo GM1
B6AF1	(C57BL/6xA/J)F1 mice
BC1	Backcross population
BTL	Binary trait loci
CFA	Complete Freund's adjuvant
CIM	Composite interval mapping
CP	Chimeric peptide
d3Tx	Thymectomy on day 3 of life
DC	Dendritic cell
FcγR	Fc γ receptor
IFA	Incomplete Freund's adjuvant
IFNγ	Interferon γ
LN	Lymph node
MHC	Major histocompatibility complex
nAOD	Neonatal AOD
NK	Natural killer
NOD	Nonobese diabetic
pZP3	Murine ZP3 peptide (330–342)
RIL	Recombinant inbred line
QTL	Quantitative trait loci
TCR	T cell receptor
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
ZP	Zona pellucida

1 Introduction

To investigate T cell immunity in chemically induced murine mammary carcinoma, Nishizuka and Sakakura depleted T cells by neonatal thymectomy and were surprised to find that the mice did not develop mammary tumors (Y. Nishizuka, personal communication 1980). The finding was reported in 1969 as induction of ovarian dysgenesis in mice thymectomized on day 3 (d3tx) but not on days 0 or 7 after birth (Nishizuka and Sakakura 1969). Mammary tumors did not occur because of failure in mammary gland development due to ovarian failure. Although the ovarian abnormality was initially inter-

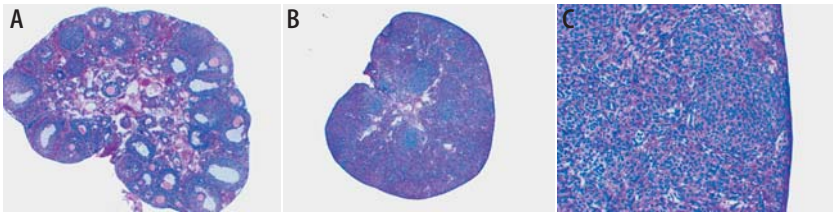

Fig. 1A–C The pathology of AOD of the d3tx mice. **A** Normal adult ovary with numerous ovarian follicles that contain growing and mature oocytes and is free of any inflammatory cells (this is also the appearance of ovaries from d3tx mice with disease suppression by half a million $CD4^+CD25^+$ T cells). **B** Ovarian atrophy in late stage of AOD, with disappearance of all oocytes and hypertrophy of interstitial gland cells. This appearance was initially called ovarian dysgenesis. Atrophy is preceded by oophoritis, or ovarian inflammation, shown in **C**. (H&E; **A** and **B**, ×50; **C**, ×200)

preted as evidence for hormonal interaction between the thymus and ovary, it was soon apparent that the ovarian change represented ovarian atrophy, the end stage of an autoimmune ovarian disease (AOD) (Fig. 1). Thus, the ovarian dysgenic changes were preceded by ovarian inflammation, the d3tx mice had autoantibody (autoAb) response to oocyte antigens (Ags) (Taguchi et al.1980; Alard et al. 2001), and AOD was adoptively transferable by spleen cells to young syngeneic recipients (Taguchi and Nishizuka 1980). Moreover, AOD was one of several autoimmune diseases attendant to d3tx in different mouse strains (Kojima and Prehn 1981). This phenomenon was subsequently confirmed by Penhale et al., who showed that adult thymectomy with fractional total body irradiation led to autoimmune disease of the thyroid and diabetes in the rats (Penhale et al. 1973 1990). Importantly, the diseases in d3tx mice and thymectomized rats were suppressed by transfer of normal adult $CD4^+$ spleen T cells (Penhale et al. 1976; Sakaguchi et al. 1982; Smith et al. 1991).

The d3tx model is a seminal milestone in autoimmunity research for at least three reasons:

1. It is a new paradigm of autoimmune disease pathogenesis—one due to perturbation of immunoregulation in normal individuals.
2. It defines suppression as an important mechanism of protection against spontaneous autoimmune disease.
3. The studies on d3tx mice, plus the data based on autoimmune disease in nu/nu mice that received $CD4^+CD5^{low}$ T cells (Sakaguchi et al. 1985; Smith et al. 1992), ultimately led to the discovery of the $CD4^+CD25^+$ T cells by Sakaguchi (1995) and Shevach (Suri-Payer et al. 1998).

For many years, the d3tx model and $CD4^+$ regulatory T cells were pursued by a handful of immunologists (Taguchi and Nishizuka 1987; Tung et al. 1987; Sakaguchi and Sakaguchi 1994; Gleeson et al. 1996; Suri-Payer et al. 1996), conducted independently of the highly-publicized but controversial $CD8^+$ suppressor T cell research initiated by Kondo and Gershon in 1970 (Gershon and Kondo 1970). The discovery of the $CD4^+CD25^+$ T cells has therefore stemmed directly from research on suppression of autoimmune diseases in the d3tx mice by normal $CD4^+$ T cells.

Since 1995, the $CD4^+CD25^+$ T cells have been defined as an important $CD4^+$ T cell functional subset, capable of regulating the innate and the adaptive immune responses, and have impact well beyond the context of autoimmunity. As described elsewhere in this monograph, many cellular, molecular, and functional properties of this regulatory T cell subset are being rapidly elucidated. In our laboratories, we have focused on the physiological function of $CD4^+CD25^+$ T cells in autoimmune disease prevention, as well as the mechanism and the genetic control of d3tx disease. We will discuss studies on the intriguing neonatal time window required for induction of AOD by d3tx and by other manipulations, summarize recent findings on the Ag specificity or Ag dependency of autoimmune disease prevention by $CD4^+CD25^+$ T cells in d3tx mice, and describe the genetic regulation of the d3tx disease.

2
Mechanism of Autoimmune Disease Induction in the Neonatal Mice

2.1
Deficiency of $CD4^+CD25^+$ Regulatory T Cells and d3tx Diseases

It has been proposed that autoimmune disease occurs in the d3tx mice because of depletion of the $CD4^+CD25^+$ T cells that have a late ontogeny (>day 5). However, the mechanism responsible for the d3tx disease is likely to be more complex because:

1. The evidence supporting this line of argument is not completely valid.
2. Disease induction is likely to depend on mechanisms besides $CD4^+CD25^+$ T cell depletion.
3. The neonatal mice have a propensity for autoimmunity for reasons besides $CD4^+CD25^+$ T cell deficiency.

It is argued that $CD4^+CD25^+$ T cell depletion is responsible for d3tx disease because autoimmune disease in the d3tx mice is suppressed by $CD4^+CD25^+$ T cells. Besides being a circular argument, it is possible that disease induction and disease suppression are phenomena that are not causally related.

For example, $CD4^+CD25^+$ T cells could inhibit disease by blocking innate inflammation rather than the Ag specific effector T cell response, as in the suppression of gastritis and colitis in *Helicobacter hepaticus*-infected mice devoid of T cells and B cells (Maloy et al. 2003). A similar argument of "two correct findings may not be related" can also be raised against the finding of $CD4^+CD25^+$ T cell suppression of disease in the athymic nu/nu mice induced by neonatal spleen T cells as evidence for neonatal deficiency of $CD4^+CD25^+$ T cells.

The $CD4^+CD25^-$ T cells are detected in the spleen of 3-day-old mice, whereas the $CD4^+CD25^+$ T cells emerge 2–3 days later, thus d3tx should enrich for effector T cells (Asano et al. 1996). This is true for the spleen; however, the lymph nodes (LNs) of normal day 3-day-old mice have the same fraction (~5%) of $CD4^+CD25^+$ cells as adult LNs (Suri-Payer et al. 1999). Neonatal LN $CD4^+CD25^+$ T cells suppress adult $CD4^+CD25^-$ T cells in vitro at a similar cell dose response as adult $CD4^+CD25^+$ T cells (Piccirillo et al. 2002; Samy and Tung, unpublished data); and recently, neonatal LN $CD4^+CD25^+$ T cells was found to suppress autoimmune disease in vivo (Samy and Tung, unpublished data). Although the $CD4^+CD25^+$ T cells transferred to adult mice are disseminated evenly in adult spleen and LNs, they preferentially home to the LNs in neonatal mice (A. Bayer and T. Malek, unpublished data). Thus differential homing of $CD4^+CD25^+$ T cells in neonatal mice may explain the different distribution of $CD4^+CD25^+$ T cells between the neonatal spleen and LNs. Because the initial T cell response in spontaneous organ specific autoimmune diseases occurs in regional LN, the cellular composition in the LN is most relevant in the regulation of the autoimmune response. Another argument in support of $CD4^+CD25^+$ T cell deficiency in d3tx mice is the finding that neonatal but not adult total spleen cells induce autoimmune disease when transferred to athymic nu/nu recipients (Smith et al. 1992; Asano et al. 1996). In retrospect, this might also be due to the selective $CD4^+CD25^+$ T cell deficiency in the spleen of neonatal cell donors.

On the other hand, autoimmune disease does not occur when the $CD4^+CD25^+$ T cells are depleted from normal mice unless accompanied by a second manipulation. For example, profound lymphopenia of d3tx mice allows expansion of pathogenic $CD4^+CD25^-$ T cells beyond the neonatal period (Min et al. 2003). Depletion of $CD25^+$ regulatory T cells from normal BALB/c adults did not cause autoimmune gastritis (AIG) unless they were injected with gastric autoAg H/K ATPase in incomplete Freund's adjuvant (IFA), which by itself is not pathogenic (for details, see the chapter by R.S. McHugh, this volume). Interestingly, $CD4^+$ T cells from the disease-free BALB/c mice with $CD4^+CD25^+$ T cell depletion were able to cause severe destructive AIG when transferred to lymphopenic nu/nu recipients (McHugh

and Shevach 2002). In Sect. 2.3, we will show that immune complex created in neonatal mice also acts as a second stimulus. Together, these studies support the concept that autoimmune disease induction and prevention are determined by competition between the effector T cell response and the regulatory T cell response, and the balance of the two cell types determines the disease vs the non-disease state (Tung 1994).

Finally, as will be described below, other autoimmune disease models have documented a neonatal predisposition to autoimmune disease independent of $CD4^+CD25^+$ T cell deficiency. These findings will be summarized in Sect. 2.3, with emphasis on a new model of AOD that could only be induced in the neonate but not the adult and is caused by maternal Ab to an ovarian Ag.

2.2 Induction of Neonatal Autoimmune Ovarian Disease and Tolerance to the Ovarian Zona Pellucida 3 autoAg and Other Self-Ags

2.2.1 Autoimmune Ovarian Disease Induction by Immunization with a ZP3 Peptide in Complete Freund's Adjuvant

ZP3 is a major glycoprotein of the ZP that surrounds growing and mature oocytes, and is accessible to circulating Abs. The immunogen is the ZP3 (330–342) peptide (pZP3), which contains a well-defined pathogenic T cell autoepitope and a distinct B cell autoepitope that induces autoAb to native ZP3 (Lou and Tung 1993). The unique features of the AOD model include the opportunity to dissect autoimmune T cell and autoAb responses, the peptide being gender-specific, and the ability to manipulate the target organ (Tung et al. 1997). For example, the duration of expression of the physiological autoAg can be examined in mice with timed ovarian ablation, implanted ovarian grafts develop normally, remain viable and functional, and serve as an Ag source as well as a target for autoimmune effector cells in mice without ovaries.

2.2.2 Neonatal Exposure to Physiological Ovary-Derived Ag Induces Tolerance, and Neonatal Immunization with Self-peptide Results in Autoimmune Disease

Neonatal mice are traditionally considered as immunologically immature, prone to development of tolerance. This is true when the tolerogen is tissue-derived. In the study on AOD induced by pZP3, adult male mice mounted a stronger T cell response than adult female mice against the female-specific Ag, and male mice developed more frequent and severe AOD in ovarian grafts. However, the differences were eliminated by ablation of endogenous

Ag (Garza et al. 2000). In a "gain of function" experiment, when male mice were engrafted with neonatal ovaries as neonates, their response to pZP3 as adults was reduced to the level of female mice (Pramoonjago et al., unpublished data). In contrast, in studies involving neonatal injection of Ag (usually of foreign origin), the neonates develop a Th2-biased response rather than tolerance (Singh et al. 1996; Garza et al. 1997; Adkins 2000). On the other hand, we have found that neonatal response to the self-peptide frequently led to autoimmune response and autoimmune disease. Indeed, neonatal mice mounted autoimmune responses and elicited autoimmune memory in situations where adult mice would be resistant (Tung et al. 2001). Thus, the nature of neonatal immune response can vary greatly depending on the nature of the antigenic stimulus.

In AOD, injection of pZP3 in IFA in neonatal female mice elicited a pathogenic autoimmune response rather than tolerance (Garza et al. 1997). AOD and ZP autoAbs were evident by 5 weeks, and subsequent challenge with pZP3 led to memory response and severe AOD. In contrast, injection of pZP3 in IFA in neonatal male mice resulted in a nonpathogenic Th2 response without AOD. Interestingly, a similar Th2 response was found in female mice whose endogenous ovarian ZP3 Ag had been surgically removed on day 2 or day 5 of life. However, Th2 deviation did not occur when the ovarian Ag was depleted at day 7 or day 14. Therefore, the neonatal immune system perceives and responds to ovarian autoAg stimulation, and the neonatal Ag exposure supports the generation of a pathogenic rather than a nonpathogenic autoimmune response. In these studies, an ovarian graft was used to monitor AOD.

2.2.3
An Environmental Factor Can Preferentially Co-stimulate Autoimmune Response and Disease in Neonatal Mice

The neonatal but not adult response to self-Ags is also uniquely modified by the environmental pinworm infection (Agersborg et al. 2001). Without pinworm infection, neonatal injection of pZP3 in water did not elicit an immune response. However, when infected with the rodent pinworm *Syphacia obvelata*, neonatal mice injected with self-pZP3 in water developed strong ZP3-specific Th2 responses and severe eosinophilic AOD, followed by a strong pathogenic Th2 memory when challenged with pZP3 in CFA. In contrast, pinworm-infected adults mounted a pathogenic Th1 response when immunized with pZP3 in CFA. Therefore, pinworm infection dramatically promotes a strong autoimmune Th2 pathogenic response; however, the effect only impacts neonatal mice.

Pinworm infection also influences the neonatal response to a peptide of the lupus Ag, Ro60 (Fig. 2). Neonatal but not adult mice, infected with rodent pinworm, produced a strong and diversified autoAb response when injected with the human Ro60 (316–335) peptide. Although adult SJL mice immunized with the Ro60 peptide (316–335) in CFA produced Abs indicative of intramolecular and intermolecular spreading (Deshmukh et al. 1999), this was not observed in adult BALB/c mice (Fig. 2D). However, as shown in Fig. 2A–C, a single injection of the Ro60 (316–335) peptide in water, in pinworm-infected neonatal BALB/c mice, induced Ab against both human and murine Ro60. In addi-

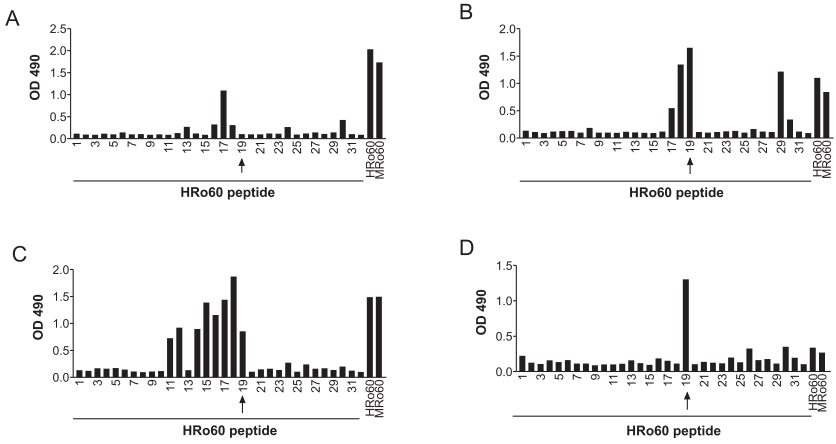

Fig. 2A–D The influence of pinworm infection on the murine antibody response to the human lupus autoantigen Ro60. Mice with pinworm infection were injected with human Ro60 peptide 316–335 (or peptide 19, *arrows*) in water. The human and murine Ro60 peptides 316–335 differ from each other by three amino acid residues. The overlapping Ro60 peptides were 20–25 amino acids long overlapped by five to ten amino acids and spanned the human Ro60. All samples used in the ELISA were diluted 1:100. **A** Reaction of serum antibody pooled from BALB/c mice 4 weeks after injection of Ro60 peptide 316–335 in water at age 2 days. The only antibody response is directed to Ro60 peptide 296–315 (peptide 17), distinct from the immunizing peptide. **B** shows the antibody response of another pinworm-infected BALB/c mouse to neonatal injection of Ro60 peptide in water, but was studied at 10 weeks. **C** The reaction of serum antibody pooled from four BALB/c mice 10 weeks following a single neonatal injection of the human Ro60 peptide 316–335 in water. **D** The response of BALB/c mice 4 weeks following a single Ro60 peptide 316–335 immunization in CFA administered in adulthood. Note that the antisera shown in **A**, **B**, and **C** also react with the recombinant Ro60 antigens; the ELISA reaction to the murine Ro60 protein was confirmed by immunoprecipitation using Ro60-associated mYRNAs derived from a radiolabeled murine cell line (data not shown)

tion, when the mice were studied at 4 weeks, they produced Ab to the Ro60 (296–315) peptide, an epitope distinct from the immunizing Ro60 (316–335) peptide (Fig. 2A). Over time, the Ab response was further diversified to additional Ro60 epitopes, indicative of intramolecular epitope spreading (Fig. 2B, 2C). The diversified Ab response occurred only in pinworm-infected neonatal mice, and this was not observed in pinworm-infected BALB/c adults and uninfected neonates (Fig. 2D). Both the pathogenic Th2 response to pZP3 and the diversified Ab response to the Ro60 peptide instantly stopped when pinworm infection was eliminated, and they resurfaced when mice were re-infected with pinworm.

These two studies document pinworm as a strong environmental factor that impacts exclusively on neonatal autoimmune response and autoimmune disease. Pinworm infection does not cause autoimmune disease per se but modulates or co-stimulates the neonatal response to self-peptide presented in a nonimmunogenic form. Moreover, in the setting of the nematode infection, pZP3 imprints a strong pathogenic Th2 memory response and stimulates a diversified B cell response. This study therefore supports the thesis of neonatal propensity to autoimmune responsiveness.

2.2.4 Neonatal Immunization Induces Autoimmune Disease Besides Autoimmune Ovarian Disease

AIG develops in neonatal rats that are injected with the gastric parietal cell H^+K^+ ATPase Ag in water (Claeys et al. 1997). Lupus autoAbs and nephritis develop in mice injected neonatally with a peptide that mimics double-stranded DNA in IFA (Singh et al. 1996). In double transgenic mice expressing influenza virus hemagglutinin and its cognate T cell receptor (TCR), a state of tolerance of the transgenic $CD8^+$ T cells is preceded by transient neonatal autoimmune response (Morgan et al. 1999). In addition, tolerance to the allogeneic lymphocytes is preceded by an early and transient graft-versus-host response to the donor MHC class II alloAg (Schurmans et al. 1991), and by a transient lupus-like disease that becomes fatal in mice with bcl-2 overexpressing B cells (Lopez-Hoyos et al. 1996).

The studies on murine AOD and other autoimmune models indicate that neonatal mice are more sensitive than adults to disease induction, and this is in turn influenced by factors including endogenous Ag expression, resistance to apoptosis, and environmental factors. We next describe the response of neonatal mice to ZP3 immune complex that results in a new intergenerational autoimmune disease known as neonatal AOD (nAOD). The nAOD model has permitted more precise dissection of the underlying mechanisms; because of

this and the relevance of the model to autoimmunity of the d3tx mice, it will be described in more detail.

2.3 The Mechanism of Neonatal Autoimmune Ovarian Disease Induced by Maternal AutoAb to ZP3

2.3.1 Neonatal Autoimmune Ovarian Disease

To investigate autoAb without concomitant T cell response, we studied a chimeric peptide (CP) that contains the foreign T cell epitope of bovine ribonuclease (94–104) and the ZP3 (335–342) native B cell epitope. The peptide (CP2) elicited strong epitope-specific Abs that bound to the ovarian ZP in vivo. Despite this, the adult ovaries were free of pathology (Lou et al. 1995). The only observable effect in adult mice was the retargeting the location of ZP3-specific Th1 or Th2-mediated tissue destruction from the ovarian interstitium to the ovarian follicles (Lou et al. 2000).

Unexpectedly, over 80% of the progenies from the ZP3-positive dams developed severe nAOD at 2 weeks of age, and 40% of those with nAOD developed ovarian atrophy, premature ovarian failure and infertility (Setiady et al. 2003) (Fig. 3). Severe nAOD was induced by serum or purified serum IgG from adult female or male mice immunized with CP2 in CFA, or by transfer of a mouse monoclonal Ab to ZP3 (335–342). Therefore, autoAb to the ZP3 (335–342) B cell epitope is sufficient to trigger severe and frequent nAOD, a process independent of maternal lymphocytes or pregnancy-associated factors.

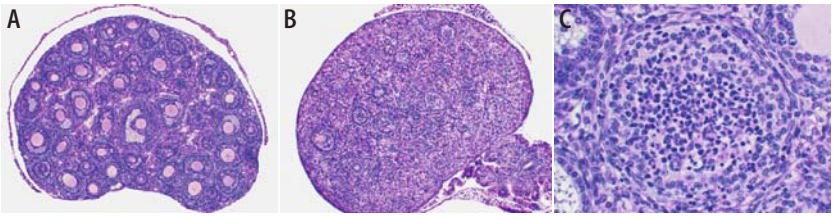

Fig. 3A–C The pathology of nAOD. **A** Normal ovary of a 2-week-old mouse, with numerous growing ovarian follicles. **B** Atrophic ovary in severe nAOD shows loss of all oocytes. **C** Ovarian inflammation has replaced the oocyte of an ovarian follicle in nAOD. (H&E; **A**, ×50; **B**, ×75; **C**, ×400)

2.3.2
Neonatal Autoimmune Ovarian Disease in the Euthymic Mice Is Mediated by De Novo Pathogenic T Cell Response

In nAOD, a 7-day interval existed between ovarian immune complex deposition and ovarian inflammation, and the inflammation was enriched in T cells and activated antigen-presenting cells (APCs) (Fig. 3, data not shown). Strikingly, when both CD4 and CD8 T cells of the neonates were depleted, the neonates did not develop nAOD. More importantly, $CD4^+$ T cells from mice with nAOD transferred severe nAOD to naive neonatal mice. Thus, maternal ZP3 autoAbs form immune complex with the endogenous Ag, and this can trigger de novo pathogenic T cell response to ovarian Ag in the neonatal mice (Setiady et al. 2003).

When neonates from untreated dams were fostered-fed milk from CP2-immunized dams, they developed high incidences and severity of nAOD when feeding commenced on day 3 or day 5 of life. However, pups fed CP2 Ab-positive milk from day 7 or day 9 did not develop nAOD. Thus frequent and severe nAOD develops only when neonatal mice are exposed to CP2 Ab within the first 5 days of life. This neonatal propensity is not due a differential rate of maternal Ab transfer in the neonatal period or to a propensity of neonatal ovaries to immune injury. When neonatal and adult ovaries were implanted under the kidney capsule of postpartum females with CP2 Ab, all the ovarian grafts contained immune complexes but they were free of AOD. In contrast, pups fostered-fed milk from the same dams developed severe nAOD. Therefore, neonatal ovaries are not uniquely prone to AOD; instead, the unique neonatal environment of days 1–5 predisposes to nAOD.

To further elucidate the unusual propensity of neonatal mice to autoimmune disease, we studied the mechanism of nAOD with respect to $CD4^+CD25^+$ T cell function and the state of innate immunity, by addressing three questions:

1. Why are older mice (>day 5) resistant to autoimmunity?
2. Why are mice more susceptible to autoimmunity during the first 5 days of life?
3. What are the cells and molecules of the neonatal innate system that are required for nAOD induction?

2.3.3
Why Are the Older Mice Resistant to nAOD?

To address whether the emergence of $CD4^+CD25^+$ regulatory T cell function could explain the resistance of the older mice to nAOD, we studied the effect of in vivo $CD25^+$ T cell depletion. Indeed, when neonatal mice were treated

with CD25 Ab and fed CP2 Ab-positive milk from postnatal day 9, 90% of them developed severe nAOD (Fig. 4A). In contrast, day 9 mice that received CD25 Ab alone were free of nAOD. Therefore, the presence of $CD4^+CD25^+$ regulatory T cell function can explain the resistance to nAOD in mice older than 7 days. As mentioned earlier (Sect. 2.1), depletion of $CD25^+$ regulatory T cells in normal mice at this age does not elicit autoimmune disease unless it is accompanied by a second event which, in this case, is autologous immune complex.

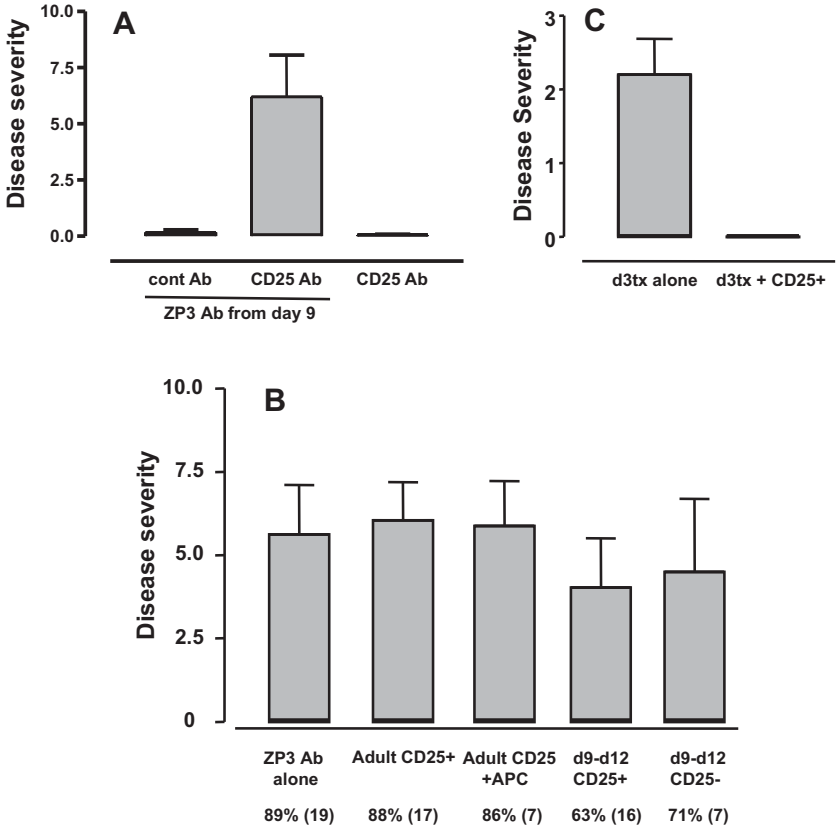

Fig. 4A–C The influence of in vivo depletion or infusion of $CD4^+CD25^+$ on nAOD development. **A** The exposure of neonatal mice to Ab to pZP3 from day 9 did not induce nAOD unless the mice were treated with Ab to CD25 (PC61); whereas CD25 Ab treatment alone did not induce nAOD. **B** The infusion of adult or d9–12 $CD4^+CD25^+$ T cells into neonatal mice did not affect disease development. The co-transfer of adult $CD4^+CD25^+$ T cells with adult APC also had no effect. **C** As control, the adult $CD4^+CD25^+$ T cells that did not affect nAOD completely inhibited AOD in the d3tx mice

2.3.4 Why Are Neonatal Mice (days 1–5) Susceptible to Neonatal Autoimmune Ovarian Disease?

If the neonatal time window of disease susceptibility is due to immaturity or preferential deficiency of $CD4^+CD25^+$ T cells, transfer of adult $CD25^+$ regulatory T cells may close the window. However, despite many attempts to prevent nAOD by infusion of $CD4^+CD25^+$ T cells from 9-day-old or adult mice, with or without co-transfer of adult APCs, we did not change the course of nAOD (Fig. 4B). These negative results suggest that the neonatal mice are resistant to suppression by $CD4^+CD25^+$ T cells. Because cells of the innate immune system [including natural killer (NK) cells, macrophages, and dendritic cells (DCs)] are known to influence adaptive immune response but also inhibit the regulatory function of $CD4^+CD25^+$ T cells (Pasare and Medzhitov 2003), we investigate the neonatal innate system in nAOD, specifically NK cells.

2.3.5 Requirement of Neonatal NK Cells, Fcγ Receptor III (FcγRIII) Positive Cells and Proinflammatory Cytokines in Neonatal Autoimmune Ovarian Disease Induction

Current knowledge on the ontogeny, phenotype, and function of neonatal NK cells is limited. In vitro studies suggest that neonatal mice have few NK cells and they are immature. Purified neonatal NK cells are barely cytotoxic against the classical NK cell targets, and do not reach adult activity until 2–3 weeks of age (Dussault and Miller 1995; Hackett, Jr. et al. 1986). The progenitors of neonatal NK cells are noted to divide more rapidly than adult NK cells (Jamieson et al. 2004). Expression of receptors for the MHC class I or class I-like molecule on neonatal NK cells is more restricted; they express predominantly CD94/NKG2A (Sivakumar et al. 1999; Kubota et al. 1999), and the Ly49 receptors are not detected before 1 week (Ortaldo et al. 2000).

We were therefore surprised to readily detect $NK1.1^+$ TCRVβ (but not $NK1.1^+$ TCRVβ^+) cells in the neonatal spleen of (C56BL/6xA/J)F1 (B6AF1) mice. The average ratio of NK cell to $\alpha\beta TCR^+$ T cells in 3-day-old mice was 0.6, which declined to 0.2 by day 9 as the T cell numbers increased. The neonatal NK cells were functional in vivo: their asialo GM1 (AGM1) positive cells, in response to lipopolysaccharide, produced as much interferon γ (IFNγ) as adult mice. Most importantly, when $NK1.1^+$ or $AGM1^+$ NK cells were depleted, the neonatal mice did not develop nAOD (Setiady et al. 2004). Neonatal NK cells are operative in both the induction and the effector phase of nAOD. Thus in adoptive transfer of nAOD, the recipient disease was ameliorated when either the donor or the recipient NK cells were depleted.

Adult NK cells can induce maturation and cytokine production by DCs, which in turn can activate naïve neonatal T cells (Ferlazzo et al. 2002; Piccioli et al. 2002; Gerosa et al. 2002; Mocikat et al. 2003; Mailliard et al. 2003). In nAOD, neonatal NK cells may function by modifying the APC function of neonatal DCs, or by stimulating T cells directly through engagement of 2B4 with CD48 on T cells (Assarsson et al. 2004). NK cell/DC interaction is bi-directional, thus both DC and T cells, when activated, can induce proliferation, activation, and cytokine production of NK cells (Fernandez et al. 1999; Ferlazzo et al. 2002; Piccioli et al. 2002; Gerosa et al. 2002; Ferlazzo et al. 2003). They may communicate by cell contact or via proinflammatory cytokines such as IFNγ and tumor necrosis factor α (TNFα) (Fernandez et al. 1999; Ferlazzo et al. 2002; Piccioli et al. 2002; Gerosa et al. 2002). Indeed, the ovaries with nAOD expressed high levels of IFNγ and TNFα that correlated with disease severity. In vivo, nAOD was inhibited by anti-IFNγ or anti-TNFα Ab. Interestingly, when cell donors were treated with IFNγ Ab, adoptively transfer of nAOD was also inhibited, thus IFNγ is likely operative during T cell induction, and NK cells a probable source of IFNγ (Setiady et al. 2004).

nAOD development is strongly influenced by the FcγR expressed on the innate cells because blockade of FcγRIIB and FcγRIII (by 2.4G2 monoclonal Ab) completely inhibited nAOD (Setiady et al. 2004). In addition, nAOD can be modulated by the stimulatory FcγRIII and the inhibitory FcγRIIb, thus the disease was ameliorated in mice deficient in FcγRIII but was greatly enhanced in FcγRIIB-deficient mice. In nAOD, the ZP3 immune complex may engage the FcγR on the NK cells, the DCs, or both. Since NK cells express predominantly FcγRIII, this may explain a dominant effect of FcγRIII deficiency in nAOD development. On the other hand, FcγRIIB and FcγRIII are co-expressed on DCs and they can potentially modulate the response of the neonatal T cells to DCs as an APC. Finally, FcγR expressed in granulocytes, monocytes, and macrophages may also contribute to nAOD through cytophilic anti-ZP3 Ab.

Many in vitro studies describe that murine neonatal T cell, neonatal NK cells, and neonatal APCs are deficient in number and function (Lu and Unanue 1982; Adkins 1999; Muthukkumar et al. 2000; Dakic et al. 2004). In contrast, the in vivo neonatal T cell and B cell responses to viral infections and vaccines are often comparable to adults (Forsthuber et al. 1996; Ridge et al. 1996; Sarzotti et al. 1996). Studies on nAOD indicate that the neonatal lymphoid compartment is far more responsive to autoantigenic stimulation than one might anticipate from the in vitro studies. Perhaps some of the discrepancies between the in vitro and in vivo findings are reconciled if the neonatal innate cells are included in the equation. In nAOD, the innate immune system including neonatal NK cells are documented to have an important role in promoting neonatal autoimmunity by enhancing neonatal APC function in other types

of immune responses. It will be important to determine whether the neonatal innate response affects $CD4^+CD25^+$ T cell function and provides another piece of the puzzle in the pathogenesis of d3tx autoimmunity.

3
Endogenous Ag Specificity and Ag Requirement for Disease Suppression by $CD4^+CD25^+$ T Cells

3.1
The Location and Ag Dependency of Suppression

To understand the physiological function of the $CD4^+CD25^+$ T cell, it is important to elucidate whether disease suppression in vivo is Ag specific. Ag specificity can have several interpretations. First, it defines the range and Ag specificity of the target cells being regulated. Does it differ in suppression of T cell subsets vs B cell and cells of the innate system (NK cells, DCs)? For example, do $CD4^+CD25^+$ T cells regulate only the T cells with shared Ag specificity, or can they cross-regulate other T cells when both cognate epitopes are presented? Second, there is the repertoire issue: how biased are the $CD4^+CD25^+$ T cells directed to self epitopes? Third, it can be the specificity of the antigenic stimulus required to expand and maintain the regulatory capacity of $CD4^+CD25^+$ T cells in the periphery, an issue important for the $CD4^+CD25^+$ T cells to maintain self-tolerance. Fourth, specificity can also imply the Ag dependency and Ag specificity during the process of regulation; and this in turn addresses the location of regulation, the source of antigenic stimulus, and the persistence of regulatory T cell action. Our recent studies have addressed the last two topics on the Ag specificity of $CD4^+CD25^+$ T cell function.

In several systems, the target organ has been found to be a site of suppression where $CD4^+CD25^+$ T cells are often co-localized with $CD4^+CD25^-$ T cells (Mottet et al. 2003; Suvas et al. 2004). On the other hand, we recently identified the regional LN as a unique site of suppression of AOD in the d3tx mice (Samy et al., unpublished data). Although the infused $CD4^+CD25^+$ T cells were widely disseminated, the ovarian draining LN was the only lymphoid organ where recipient $CD4^+$ T cell response was completely inhibited. This finding implies that suppression of AOD by polyclonal $CD4^+CD25^+$ T cells depends on stimulation of the regulatory T cells by endogenous Ags. This is also supported by an earlier study that documented the critical requirement of endogenous ovarian Ag for maintenance of the physiological tolerance state. As mentioned earlier (Sect. 2.2.2), the supremacy of male over female

response to the female specific self-Ag pZP3 indicates female mice are tolerant to ZP3 and this was terminated by ovarian ablation (Garza et al. 2000). In addition, continuous Ag stimulation was found to be required to maintain tolerance, which was terminated within 1 week after ovarian ablation. In our recent study on suppression of AOD in d3tx mice, depletion of the input $CD4^+CD25^+$ T cells also promptly led to emergence of severe AOD (Samy et al., unpublished data). Similar reversibility of suppression has been reported in experimental autoimmune encephalomyelitis (EAE) induced by T cells with transgenic TCR to myelin basic protein (Hori et al. 2002). The requirement of persistence of $CD4^+CD25^+$ T cells in suppression of autoimmunity argues against the importance of clonal elimination of effector T cells or induction of infectious tolerance.

3.2 Suppression of Autoimmune Disease by Regulatory Cells from Donors with or Without the Relevant Self-Ag

Earlier studies reported that AOD in the d3tx mice was suppressed by thymic graft or spleen cells from female mice, whereas male thymic cells and male spleen cells either do not suppress AOD or only in excess cell numbers (Nishizuka and Sakakura 1969; Sakaguchi et al. 1982). Our attempt to reproduce this finding was not successful (Smith et al. 1991). More recently, we have confirmed that our result was correct by showing that $CD4^+CD25^+$ T cells from male and female donors suppressed AOD equally, with identical cell dose responses (Setiady et al., preliminary data).

In view of differential suppression of autoimmune prostatitis (AIP) and autoimmune thyroiditis by T cells from Ag-positive vs Ag-negative cell donors (described below), how do we explain their equal suppression of AOD? Our interpretation is that even if the regulatory capacities of male and female $CD4^+CD25^+$ T cells for AOD suppression are different, they are equalized when the cells encounter the endogenous ovarian Ag in the young d3tx host. Indeed, we have shown that ovarian Ags (mater and ZP3) are expressed from birth and have the capacity to stimulate T cells on day 3 (Alard et al. 2001). This is also exemplified by the process of diversified autoAb response that depends on de novo B cell response to endogenous ovarian Ag. Immunized female mice with a ZP3 peptide that contains T but not native B epitope (in CFA) elicited Ab response to a distant native ZP3 B cell epitope within 7 days, 2 days after detectable response to the ZP3 T cell epitope (Lou et al. 1996). Other examples of endogenous ovarian antigenic stimulation, mentioned in Sect. 2.2.2, are:

1. The endogenous Ag requirement (in days 1–5) in promoting pathogenic Th1 response attendant to neonatal stimulation by pZP3 in IFA.
2. The rapid termination of female tolerance to pZP3 within 1 week of ovarian ablation.

Because of the highly accessible ovarian Ags, the AOD model may not be suitable for differentiating the regulatory capacity of $CD4^+CD25^+$ T cells from Ag-positive vs Ag-negative donors. Indeed, more clear-cut results have come from studies on autoimmune thyroiditis and AIP.

Seddon and Mason (1999) studied total $CD4^+$ T cells in suppression of autoimmune thyroiditis in nu/nu rats induced by the $CD4^+CD46RC^{high}$ effector T cells. Using a single cell dose, suppression was evident only when the $CD4^+$ T cells came from euthyroid donors, whereas autoimmune diabetes was suppressed by cells from both euthyroid and athyroid donors.

In murine autoimmune prostatitis, in which prostate Ags are expressed at the age of 2 weeks, it was found that total male spleen cells suppressed better than female cells (Taguchi and Nishizuka 1987). The male supremacy was lost when the cell donors were neonatally orchiectomized to prevent prostate development, but it was restored when prostate development was subsequently induced by dihydrotestosterone. We have confirmed this interesting finding by showing that $CD4^+CD25^+$ T cells from male donors also suppressed more efficiently than cells from female donors (Setiady et al., unpublished data). Importantly, exposure of cell donors to endogenous Ag for only 10 days was sufficient to enhance the regulatory capacity of $CD4^+CD25^+$ T cells of Ag-negative donors to that of Ag-positive donors. This finding is relevant to AOD suppression. For example, for the inexperienced male cells to rapidly gain regulatory capacity through encounter with the ovarian Ag in the d3tx recipients, it may need to occur before effector T cell activation at 2–3 weeks (Alard et al. 2001). On the other hand, this would not be possible in AIP suppression because of the late ontogeny of prostate Ag expression.

Taken together, the in vivo studies in d3tx mice support Ag-specific suppression of autoimmune diseases by $CD4^+CD25^+$ T cells, though the findings do not rule out additional suppression by nonspecific means. Our study on Ag specificity further emphasizes the dynamic nature of immune suppression by the $CD4^+CD25^+$ T cells:

1. The regulatory T cell function is critically dependent on their persistent stimulation by endogenous Ag.
2. Effective disease suppression (or tolerance) is critically dependent on the persistence of the $CD4^+CD25^+$ T cells in the host.

These important findings will influence the design of immunotherapy based on $CD4^+CD25^+$ T cells.

4
Genetic Control of Susceptibility to D3tx-Induced Autoimmune Disease

4.1
Genetic Studies on Inbred Strains of Mice

Kojima and Prehn's study (1981) examining susceptibility to d3Tx-induced autoimmune disease in 21 different inbred and congenic strains of mice found strain variation in organ involvement, incidence, and severity of disease; they also found that AIP was the only disease with a clear *H2* association. However, *H2*-linkage has subsequently been extended to include susceptibility to both AOD and AIG (Silveira et al. 2001; Roper et al. 2002). Additional studies were carried out to address the inheritance of d3tx-induced autoimmune disease. The results obtained using reciprocal F1 hybrid, backcross, and F2 intercross populations are consistent with oligogenic control by a limited number of interacting loci. Importantly, however, they revealed that susceptibility to AOD exhibits a maternal parent-of-origin effect in that the incidence of disease observed in F1 hybrid mice is significantly greater when the dam is the susceptible parental strain (Kojima and Prehn 1981). Preliminary attempts to map the genes controlling susceptibility to AOD, AIG, and AIP utilizing recombinant inbred lines (RIL) derived from BALB/cByJ and C57BL/6ByJ mice suggested a possible association of AIG with the minor histocompatibility locus *H27*, whose map location is unknown, and again, AIP with *H2*.

4.2
Mapping Loci Controlling Susceptibility to D3Tx-Induced Autoimmune Disease

There is little doubt that transgenic and gene knockout technologies provide insight into identifying genes involved in various aspects of immune processes (Yeung et al. 1993; Fischer and Malissen 1998). However, less is known about the function of a particular molecule as it pertains to its larger ecologically relevant and evolutionarily selected role in the immune system. Such information can only be obtained by identifying and characterizing the naturally occurring, evolutionarily selected alleles giving rise to phenotypic variation.

AOD and AIG are amenable to forward genetic analysis based on disease incidence (Kojima and Prehn 1981; Tung et al. 1987; Silveira et al. 1999). Approximately 90% of d3Tx female A/J and B6AF1 hybrid mice develop AOD and 80% of BALB/cCrSlc mice develop AIG with C57BL/6 J mice exhibiting less than 10% disease. Genome scans and linkage analyses carried out using mapping populations segregating susceptibility to AOD, AIG, and their component phenotypes are consistent with genetic control by a limited number of disease genes rather than polygenic inheritence. A summary of the binary

trait loci (BTL) and quantitative trait loci (QTL) controlling susceptibility to AOD and AIG and their component phenotypes is presented in Table 1.

Genetic analysis of AOD utilizing a (C57BL/6 J × A/J) × C57BL/6 J backcross population (BC1) initially indicated that susceptibility was controlled by a single dominant locus (*Aod1*) with the results of the initial genome scan placing *Aod1* on central chromosome 16 (Wardell et al. 1995). Subsequently, *Aod2*, a second locus associated with susceptibility to ovarian atrophy was mapped to chromosome 3 (Teuscher et al. 1996). These studies, however, focused on susceptibility to AOD as a binary trait (affected vs. unaffected).

Composite interval mapping (CIM) -based QTL analysis (Zeng 1993, 1994), utilizing semi-quantitative histopathological lesion scores for oophoritis and atrophy as well as anti-ovarian autoantibody (AOA) titers, verified *Aod1* and *Aod2*; and identified three new QTL involved in AOD; *Aod3* (Chr. 1), *Aod4* (Chr. 2) and *Aod5* (Chr. 7) (Roper et al. 2002). CIM-QTL analysis using the A × B and B × A RILs also verified *Aod3* and detected linkage to *H2.* Importantly, statistical genetic-based interaction analysis (Wendell and Gorski 1997; Roper et al. 1999) also predicted the existence of epistasis between *Aod1–5*, *Gasa2*, a QTL controlling d3Tx-induced AIG (Silveira et al. 1999, 2001), and with *H2* (Table 2). For example, *Aod3* was predicted to interact with *Gasa2*. Similar results were observed for AOD with *Aod3* and *Aod5*, *Aod3* and *H2,* and *Aod1* and *Aod4* and together explained 35.8% of the AOD trait variance (Roper et al. 2002).

As the first step toward positionally cloning *Aod1*, we generated a panel of interval-specific bidirectional recombinant congenic lines encompassing the genetic interval on chromosome 16 (Roper et al. 2003). The results of these studies indicated that *Aod1* does control AOD but rather than being a single locus, *Aod1* is comprised of two linked QTL with opposing allelic effects. *Aod1a* resides between *D16Mit211* (23.3 cM) and *D16Mit51* (66.75 cM) on chromosome 16, whereas *Aod1b* maps proximal of *Aod1a* between *D16Mit89* (20.9 cM) and *D16Mit211* (23.3 cM).

A similar genetic analysis for AIG was carried out using a (BALB/cCrSlc × C57BL/6) F2 intercross population (Silveira et al. 1999). Two linked QTL on telomeric chromosome 4, *Gasa1* at ~60–70 cM and *Gasa2* at ~78–82 cM, were implicated in the genetic control of susceptibility to AIG, as assessed by the existence of histopathological lesions and H^+/K^+ ATPase-specific autoAb titers. A subsequent study utilizing partitioned Chi-square analysis revealed the existence of two additional QTL controlling susceptibility to AIG: *Gasa3* on chromosome 6 at ~42–49 cM and *Gasa4* (*H2)* (Silveira et al. 2001). Potential epistatic interactions between the QTL controlling susceptibility to AIG were also implicated in susceptibility to AIG, i.e., *Gasa2* × *Gasa4* (*H2*).

The detection of epistasis among and between the QTL controlling AOD and AIG as well as with *H2* (Table 2) suggest that the QTL controlling d3Tx-

Table 1 Summary of QTL controlling d3Tx-induced autoimmune diseases identified to date

Disease	Locus	Cross	Chr.	Marker(s)	cM	Phenotype	Reference
AOD	*Aod1*	BC1	16	*D16Mit58-D16Mit59*	23–28	Oophoritis	Wardell et al. 1995; Roper et al. 2002
	Aod1a	Congenic	16	*D16Mit211-D16Mit51*	23.3–66.8	Oophoritis	Roper et al. 2003
	Aod1b	Congenic	16	*D16Mit89-D16Mit211*	20.9–23.3	Oophoritis	Roper et al. 2003
	Aod2	BC1	3	*D16Mit21-D16Mit94*	19–22	Atrophy	Teuscher et al. 1996; Roper et al. 2002
	Aod3	BC1	1	*D1Mit417*	63	Oophoritis	Roper et al. 2002
		BC1	1	*D1Mit45*	58	Atrophy	Roper et al. 2002
		RIL	1	*D1Mit128*	37	Oophoritis	Roper et al. 2002
	Aod4	BC1	2	*D2Mit452*	79	Atrophy	Roper et al. 2002
	Aod5	BC1	7	*D7Mit340-D7Mit77*	1–7	Auto-Ab	Roper et al. 2002
	Aod6 (*H2*)	RIL	17	*D17Mit62*	17.4	Oophoritis	Roper et al. 2002
AIG	*Gasa1*	F2	4	*D4Mit203-D4Mit284*	60–70	Gastritis	Silveira et al. 1999
	Gasa2	F2	4	*D4Mit127-D4Mit344*	78–82	Gastritis, auto-Ab	Silveira et al. 1999
	Gasa3	F2	6	*D6Mit67-D6Mit287*	42–49	Gastritis	Silveira et al. 2001
	Gasa4 (*H2*)	Congenic	17		19	Gastritis	Silveira et al. 2001

Table 2 Interaction of QTL among AOD and AG phenotypes

Phenotype	Trait-specific QTL[a]	Interacting QTL[b]	% Variance[c]	*F*	*P* value
Oophoritis			35.8	6.2	< 0.0001
	Aod1	*Aod3* × *Gasa2*			
	Aod3	*Aod3* × *Aod5*			
		Aod3 × *Aod6* (*H2*)			
		Aod1 × *Aod4*			
Atrophy			43.6	9.3	< 0.0001
	Aod2	*Aod1* × *Aod4*			
	Aod3	*Aod2* × *Aod6* (*H2*)			
	Aod4				
AOA			13.9	3.6	0.0180
	Aod5	*Aod2* × *Aod4*			
Gastritis					
	Gasa1	*Gasa2* × *Gasa4* (*H2*)	ND[d]	ND	ND
	Gasa2				
	Gasa3				
	Gasa4				

[a] Independent variables in linear regression model represented by loci identified by CIM.
[b] Independent variables that represent significant interactions as found by stepwise selection.
[c] Variance, *F* and *P* values are for entire model with all terms included in the linear regression model.
[d] Not determined.

induced autoimmunity may be both organ-specific and more generalized in their effects with respect to the genesis and activity of the immunoregulatory mechanisms maintaining peripheral tolerance. The non-MHC-linked "shared" autoimmune disease gene hypothesis, first proposed by Teuscher in 1985 (Teuscher 1985; Sudweeks et al. 1993; Meeker et al. 1995), was recently validated by our identification of *Bphs* as *Hrh1*, a "shared" gene in EAE and autoimmune orchitis (Ma et al. 2002). Additionally, given the role of $CD4^+CD25^+$ regulatory T cells in d3Tx-induced diseases, it is likely that one or more of the QTL controlling d3Tx-induced autoimmune disease play a role in the genesis and maintenance of these cells or in controlling their effector functions.

4.3
Positional-Candidate Genes for AOD and AIG QTL That Are Differentially Expressed by $CD4^+CD25^+$ Regulatory T Cells

The pathway to gene discovery using the positional-candidate gene approach involves genetic mapping of trait loci; physically delineating a support interval for each locus by congenic mapping; gene identification using expression or structural polymorphisms to guide the selection from a list of candidate genes within the interval; and tests for expression of that gene in relevant cells, the mechanism of its action, and the way that natural alleles of the gene shape its behavior, all in the context of environmental influences. In this scheme, candidate gene selection is primarily based on phenotype-genotype relationships delineated by congenic mapping. However, additional criteria can be used to aid in considering a particular gene, or set of genes, as potential candidates when congenic mapping-based genotype–phenotype relationships are unavailable (Abiola et al. 2003). For example, genes residing within the AOD and AIG BTL and QTL intervals that exhibit differential expression in $CD4^+CD25^+$ regulatory T cells are promising candidates for initial evaluation.

Comparative microarray analyses between $CD4^+CD25^+$ T cells and other T cells in several different models resulted in the identification of a limited set of differentially expressed genes (Bystry et al. 2001; Lechner et al. 2001; Gavin et al. 2002; McHugh et al. 2002; Graca et al. 2002; Zelenika et al. 2002). To identify which of these genes map within the genetic intervals encompassing the AOD and AIG disease susceptibility loci we determined their map locations by searching the MGI and NCBI linkage maps. The locations for the unmapped genes were determined by locating their sequence within the mouse genome using the ENSEMBL or UCSC genome browsers and identifying the closest linked gene or marker whose map location was known. The list of genes that was identified in this way is presented in Table 3. Surprisingly, a number of the differentially expressed genes mapped within the genetic intervals encompassing the AOD and AIG susceptibility loci. Importantly, there were no differentially expressed loci that mapped within the *Aod1a*, *Aod1b* and *Gasa1* intervals, suggesting that the detected occurrences are not simply random events. Additionally, it is worth nothing that several of the associations co-localize with QTL involved in other autoimmune diseases (http://www.informatics.jax.org/) in which $CD4^+CD25^+$ regulatory T cells have been implicated (multiple reviews in Parham 2001). Thus, structural- or expression-level polymorphism in these genes could underlie "shared" autoimmune disease susceptibility loci.

Of the genes exhibiting differential expression in $CD4^+CD25^+$ regulatory T cells several were identified in more than one study (Table 3, highlighted in bold type). These include *Pdcd1* (programmed cell death-1); *Tnfrsf1b*

Table 3 Summary of positional-candidate genes that are differentially expressed by $CD4^+CD25^+$ T-cells mapping within the genetic intervals encompassing QTL controlling AOD and AG

QTL	Differentially expressed positional candidate genes			
	Chr.	cM[a]	Gene designation[b]	Process/functional category[c]
Aod2	3	19	*Il2*	Cell proliferation, cellular defense response, IL-2 receptor activity
Aod3	1	54–59	***Pdcd1***	Apoptosis
Aod4	2	81	*Tde1*	Induction of apoptosis
Aod3×*Gasa2, Gasa1,*	4	76	***Tnfrsf1b***	Cell proliferation, cell surface receptor linked signal transduction
Gasa2, Gasa2 × *Gasa4 (H2)*		76	***Tnfrsf9***	Defense response
		79	***Tnfrsf4***	Cellular defense response
		79	***Tnfrsf18***	Receptor activity
Aod5	7	3	*Pira1*	Unknown
		4	*Apoe*	Lipid transport, lipoprotein metabolism
		7	***Tgfb1***	Cell growth, cell proliferation
Aod6, Gasa4 (H2)	17	16	*Pim1*	Cell growth and/or maintenance, protein amino acid phosphorylation
		19	***Psmb9***	Protein metabolism, proteasome complex
		19	*Psmb8*	Immune response, ubiquitin-dependent protein catabolism, endopeptidase
		19	***Lta***	Cell growth and/or maintenance, cell proliferation
		19	***Ltb***	Lymph gland development
		20	*H2-M3*	Defense response

[a]Locations are based on the MGI linkage map (http://www.informatics.jax.org/). Map locations for unmapped EST were determined by BLAST analysis and placed according to the closest linked, mapped marker or gene.

[b]Enboldened genes are those that appeared in two or more comparisons (Bystry et al., 2001; Lechner et al., 2001; Gavin et al., 2002; McHugh et al., 2002; Graca et al., 2002; Zelenika et al., 2002).

[c]Process and functional classifications are according to MGI (http://www.informatics.jax.org/).

(tumor necrosis factor receptor superfamily, member 1b); *Tnfrsf9* (tumor necrosis factor receptor superfamily, member 9); *Tnfrsf4* (tumor necrosis factor receptor superfamily, member 4); *Tnfrsf18* (tumor necrosis factor receptor superfamily, member 18); *Tgfb1* (transforming growth factor, beta 1); *Psmb9* (proteosome subunit, beta type 9); *Lta* (lymphotoxin A); and *Ltb* (lymphotoxin B).

4.3.1 Pdcd1 as a Candidate for Aod3

Pdcd1, an inhibitory co-stimulatory receptor induced on activated T, B, and myeloid cells, plays a role in the regulation of peripheral tolerance in that Pdcd1 signaling in T cells induces anergy (Okazaki et al. 2002; Leibson 2004). Disruption of *Pdcd1* also leads to strain-specific autoimmune phenomena, i.e., C57BL/6 *Pdcd1*$^{-/-}$ mice develop spontaneous lupus-like disease, whereas BALB/c *Pdcd1*$^{-/-}$ mice exhibit autoAb-mediated dilated cardiomyopathy (Nishimura et al. 1999, 2001; Okazaki et al. 2003). In addition, *Pdcd1* has been implicated in the regulation of both autoimmune diabetes (Ansari et al. 2003) and EAE (Salama et al. 2003). The role of *Pdcd1* in the genesis and/or function of $CD4^+CD25^+$ regulatory T cells is unclear but it was recently shown to be upregulated on $CD4^+CD25^+$ regulatory T cells generated by exposure of $CD4^+$CD25- T cells to TGFβ (Park et al. 2004). It has also been shown to play a role in thymocyte development (Nishimura et al. 2000). Most importantly, with respect to *Pdcd1* as a candidate for *Aod3*, polymorphism in *Pdcd1* has been reported to be associated with susceptibility to systemic lupus erythematosus (Prokunina et al. 2002), type I diabetes (Nielsen et al. 2003), and rheumatoid arthritis (Prokunina et al. 2004).

4.3.2 The Tumor Necrosis Factor Receptor Superfamily Genes

Of the tumor necrosis factor receptor superfamily genes that are candidates for *Gasa1*, *Gasa2*, *Aod3* (based on the interaction between *Aod3* and *Gasa2*) (Roper et al. 2002), and the interaction between *Gasa2* and *Gasa4* (*H2*) (Silveira et al. 2001), *Tnfrsf18/Gitr* is of particular note. Depletion of $Tnfrsf18^+$ cells or stimulation of Tnfrsf18 was shown to abrogate $CD4^+CD25^+$ regulatory T cell activity resulting in the development of autoimmune disease (Shimizu et al. 2002; McHugh et al. 2002).

4.3.3
Tgfb1

Tgfb1 is a pleiotropic factor that plays a central function in maintenance of immune homeostasis (reviewed in Letterio and Roberts 1998) and several studies suggest a possible link between *Tgfb1* and regulatory T cells (Nakamura et al. 2001; Yamagiwa et al. 2001). It has been suggested that TCR activation in the presence of Tgfb1 converts naïve mouse $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ regulatory T cells through the induction of Foxp3 (Chen et al. 2003; Schramm et al. 2004), a gene that has been proposed to be a master switch for $CD4^+CD25^+$ regulatory T cell development and function (Hori et al. 2003; Fontenot et al. 2003; Khattri et al. 2003; reviewed in Fehervari and Sakaguchi 2004). Interestingly, it was recently reported that Tgfb1 co-stimulation of $CD4^+CD25^-$ T cells leads to an increase in the level of Pdcd1 expression upon conversion to $CD4^+CD25^+$ regulatory T cells (Park et al. 2004). The existence of this amplification loop may reflect the epistatic interaction observed between *Aod3* and *Aod5* in the genetic control of oophoritis (Table 2).

4.3.4
H2

Genetic linkage of autoimmune disease susceptibility to the MHC is believed to reflect class I- and class II-based genetic restriction of autoantigenic peptide presentation to T cells (Rhodes and Trowsdale 1999; Sonderstrup and McDevitt 2001; Fourneau et al. 2004). However, the existence of other MHC-linked genes functioning in susceptibility to autoimmune and infectious diseases is becoming increasingly evident (Hattori et al. 1999; Morel et al. 1999; Boulard et al. 2002a; Teuscher et al. 2004). *Psmb9*, *Lta* and *Ltb*, the three *H2*-linked genes differentially expressed in $CD4^+CD25^+$ regulatory T cells have all received considerable attention as candidates for MHC-linked autoimmune disease susceptibility genes. *Psmb9* is known to have three structural alleles, $Psmb9^d$, $Psmb9^{b,}$ and $Psmb9^q$ that correlate with the *H2* haplotypes of various inbred strains of mice (Zhou et al. 1993). The $Psmb9^d$ (A/J and BALB/cCrSlc allele) and $Psmb9^b$ (C57BL/6 J) alleles are segregating in both the AOD and AIG BC1 populations. Ltb has been shown to affect the function of *aire* (Chin et al. 2003). The mutation of *aire* alone has been shown to result in human autoimmune polyglandular syndromes type I (APECED) (Ruan and She 2004), and mice with targeted deletion of *aire* develop autoimmune disease of the stomach, ovary, and eye (Anderson et al. 2002), the typical autoimmune diseases that develop in d3tx mice.

4.3.5 Il2

Of the genes exhibiting differential expression in $CD4^+CD25^+$ regulatory T cells, *Il2* is a particularly strong candidate for a "shared" autoimmune disease susceptibility gene. *Il2* was originally identified as a candidate for *Aod2*, and based on its co-localization with *Idd3*, the strongest QTL associated with resistance to IDDM in the NOD mouse (Lyons et al. 2000; Podolin et al. 2000; Ikegami et al. 2002; Ikegami et al. 2003), we hypothesized that a structural polymorphism in *Il2* (Chesnut et al. 1993) may reflect a "shared" autoimmune disease susceptibility gene underlying the two QTL (Teuscher et al. 1996). Subsequent studies also implicated *Il2* as a candidate for *eae3/20* (Butterfield et al. 1998; Encinas et al. 1999) and *Ssial2*, controlling autoimmune sialoadenitis in NOD mice (Boulard et al. 2002b). Importantly, recombinant IL2 allelic proteins have been reported to differentially influence IL2 regulated responses (Matesanz and Alcina 1996, 1998; Choi et al. 2002). IL2 expression at the mRNA level also differs between EAE-susceptible SJL/J and EAE-resistant B10.S/DvTe $CD4^+$ T cells following stimulation with anti-CD3/CD28 monoclonal Ab (unpublished data). Thus, IL2 is a candidate gene based on the existence of both a structural- and expression-level polymorphism. Interestingly, a sequence polymorphism in the human *Il2* promoter (G/T and T/T) at −330 (–384 from the ATG), influencing IL2 synthesis, has been reported to be associated with susceptibility to multiple sclerosis (Matesanz et al. 2001, 2004).

Support for *Il2* as a candidate gene in the genesis, maturation, and maintenance of $CD4^+CD25^+$ regulatory T cells is based on the differential expression of IL2 and IL2ra between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells after stimulation with anti-CD3 and IL2 (McHugh et al. 2002); *Il2*-knockout (*Il2*KO), *Il2ra*KO (CD25), and *Il2rb*KO mice all develop autoimmune phenomenon (Sadlack et al. 1993; Willerford et al. 1995; Suzuki et al. 1995), with 25%–50% of *Il2*KO and *Il2ra*KO mice dying from severe hemolytic anemia and the remaining mice developing wasting disease (Sadlack et al. 1993; Willerford et al. 1995); *Il2ra*KO mice lack functional $CD4^+CD25^+$ regulatory T cells (Furtado et al. 2002); adoptive transfer of normal $CD4^+CD25^+$ T cells into neonatal *Il2rb*KO mice prevents autoimmunity (Malek et al. 2002); autoimmunity seen in *Il2rb*KO mice can be prevented by selectively expressing *Il2rb* in the thymus (Malek et al. 2000); $CD4^+CD25^+$ regulatory T cells "de-anergized" by stimulation with high levels of IL2 lose their capacity to suppress disease (Takahashi et al. 1998); and $CD4^+CD25^+$ regulatory T cells constitutively express CD25 (Sakaguchi et al. 1995). These observations suggest that IL2–IL2r signaling plays an essential role in the genesis, maturation, and maintenance of $CD4^+CD25^+$ regulatory T cells mediating peripheral tolerance (Malek 2003;

Nelson 2004) and underscores the concept that the *Il2* polymorphisms may have selectively unique ontogenic effects within the thymus during the genesis and selection of these cells and in the periphery during their maturation and maintenance of regulatory activity.

4.3.6
Positional-Candidate Genes for AOD and AIG QTL

Immunologically relevant positional-candidate genes for *Gasa3* on chromosome 6 have yet to be identified within the linkage interval (http://www.informatics.jax.org/). Similarly, given the current size of the interval encompassing *Aod1a*, identification of potential candidates is highly speculative. However, *Il10rb* (interleukin-10 receptor β) at 61 cM is an intriguing candidate since IL10 has been implicated in the establishment and maintenance of $CD4^+CD25^+$ T cells (Annacker et al. 2001). *Trfr* (transferrin receptor or Cd71) at 21.2 cM is a potential candidate for *Aod1b*. Trfr is downregulated during adult T cell development as well as in ontogeny prior to the appearance of the α/β TCR and therefore serves as a marker of immature, proliferating T cells (Brekelmans et al. 1994). *Stfa1*, *Stfa2*, *Stfa3* (stefin A1, A2 and A3) at 22.85 cM are inhibitors of cysteine endo- and exopeptidases (Bode and Huber 2000) such as cathepsin L and S involved in Ag processing (Pluger et al. 2002; Hsieh et al. 2002). Most importantly, cathepsin S inhibitors were shown to prevent autoAg presentation in vitro, and in vivo treatment with cathepsin inhibitors blocks lymphocytic infiltration into the salivary and lacrimal glands, abrogates autoAb production, and promotes the recovery from autoimmune disease in these organs in d3tx NFS/sld mice (Saegusa et al. 2002). These polymorphisms have the potential of functioning at both the selection phase of $CD4^+CD25^+$ T cells during thymopoiesis and their maturation and maintenance within the periphery (Parham 2001; Fehervari and Sakaguchi 2004), and at the effector or inflammatory phase of the disease mediated by $CD4^+CD25^-$ effector T cells. However, to date, the polymorphic residues of *Stfa1* and *Stfa2* have not been modeled with respect to their functionality as inhibitors of cathepsin activity.

4.3.7
The Autoantigen in d3tx-Induced Autoimmune Ovarian Disease

A potential candidate gene within the *Aod5* interval is *Nalp5* (NACHT, leucine-rich repeat and PYD containing 5; also known as *Mater*, *Op1*, and PAN11). *Nalp5* is an ovarian specific autoAg identified by its reactivity with autoAb present in the sera of d3tx mice (Tong and Nelson 1999). We sequenced

Nalp5 and identified it as a structurally polymorphic candidate gene for *Aod5* (Roper et al. 2003). Importantly, sequencing results from other strains of mice that exhibit differential susceptibility to AOD also express the same polymorphic splice variants (unpublished data). The polymorphic peptides arising from the A/J and C57BL/6 J Nalp5 alleles may affect the genesis, maturation, and maintenance of $CD4^+CD25^-$ effector T cells, $CD4^+CD25^+$ regulatory T cells or both, and thereby directly impact disease susceptibility. $CD4^+CD25^+$ regulatory T cells appear to be selected from a cellular pool with different affinities compared to regulatory T cells that are CD25 negative (Suto et al. 2002); and our studies described in Sect. 3 on the requirement for self-Ags in the generation and maintenance of $CD4^+CD25^+$ T cells is consistent with this possibility. Moreover, any polymorphism in the ontogeny of autoAg expression during the first few days of life may also strongly influence disease susceptibility in d3tx mice.

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