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Effector Mechanisms of the Autoimmune Syndrome in the Murine Model of Autoimmune Polyglandular Syndrome Type 1

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Mutations in the Aire gene result in a clinical phenomenon known as Autoimmune Polyglandular Syndrome (APS) Type I, which classically manifests as a triad of adrenal insufficiency, hypoparathyroidism, and chronic mucocutaneous infections. In addition to this triad, a number of other autoimmune diseases have been observed in APS1 patients including Sjögren’s syndrome, vitiligo, alopecia, uveitis, and others. Aire-deficient mice, the animal model for APS1, have highlighted the role of the thymus in the disease process and demonstrated a failure in central tolerance in aire-deficient mice. However, autoantibodies have been observed against multiple organs in both mice and humans, making it unclear what the specific role of B and T cells are in the pathogenesis of disease. Using the aire-deficient mouse as a preclinical model for APS1, we have investigated the relative contribution of specific lymphocyte populations, with the goal of identifying the cell populations which may be targeted for rational therapeutic design. In this study, we show that T cells are indispensable to the breakdown of self-tolerance, in contrast to B cells which play a more limited role in autoimmunity. Th1 polarized CD4+ T cells, in particular, are major contributors to the autoimmune response. With this knowledge, we go on to use therapies targeted at T cells to investigate their ability to modulate disease in vivo. Depletion of CD4+ T cells using a neutralizing Ab ameliorated the disease process. Thus, therapies targeted specifically at the CD4+ T cell subset may help control autoimmune disease in patients with APS1. The Journal of Immunology, 2008, 181: 4072–4079.

A
toimmune Polyglandular Syndrome (APS)3 Type I is a monogenic autoimmune disease that is inherited in an autosomal recessive pattern (1). Individuals with this disease present with a clinical triad including adrenal insufficiency, chronic mucocutaneous infections, and hypoparathyroidism. In addition to these hallmarks of disease, patients frequently have other autoimmune manifestations including type 1 Diabetes, Sjögren’s syndrome, vitiligo, keratitis, alopecia, gastritis, and other syndromes believed to be of an autoimmune etiology (2).

The basis for such a strong predisposition to autoimmunity was genetically mapped by two independent groups, resulting in the identification of the AIRE gene (3, 4). The Aire protein, which bears strong resemblance to a transcription factor and has been shown to localize to nuclear speckles (5), is expressed in a subset of medullary thymic epithelial cells (mTECs) that are associated with negative selection of developing thymocytes. Within mTECs, Aire controls the promiscuous expression of many peripheral autoantigens through mechanisms that are not completely understood (6). The absence of Aire expression results in an inability to remove autoreactive thymocytes from the immune repertoire, ultimately resulting in autoimmune disease against multiple tissues (7).

Despite the evidence suggesting the thymus as the key to the initiation of the disease process, multiple cells could play a role in the autoimmunity that eventually ensues and tissue destruction may be mediated by cell types other than T cells. In APS1 patients and aire-deficient mice, autoantibodies recognizing several organ-specific autoantigens have been identified including insulin, glutamic acid decarboxylase, cytochrome P450, 21-hydroxylase (8), and more recently tudor-domain containing protein 6 in humans (9) as well as interphotoreceptor retinoid binding protein (7), fodrin (10), pancreas-specific protein disulfide isomerase (11), and mucin-6 (12) in aire-deficient mice. It is unclear, however, whether or not these autoantibodies are pathogenic and what role they, or the B cells that produce them, may play in the progression of disease.

Aire-deficient mice remain the best tool available to study this unique process and mimic the human disease in many ways. Due in part to the difficulties in studying human patients and their relative rarity in clinical medicine, little is known about the specific contribution of different cell types in disease pathogenesis and progression. To further understand the role that the immune system plays in aire-mediated autoimmunity, we performed a detailed analysis of lymphocyte function within aire-deficient mice and bred the aire mutation onto several genetic backgrounds including mice deficient for T and B cells. In this study, we present the results of these studies on the relative role of T and B cells in mediating disease and demonstrate that T cells are indispensable to the disease process, whereas B cells play a more limited role in...
autoimmunity. Therapies targeting CD4+ T cells ameliorated autoimmunity, supporting these genetic and adoptive transfer studies and suggesting a clinically relevant avenue of therapeutic exploration.

Materials and Methods

Mice
Aire-deficient mice were generated as previously described (6) and were backcrossed into the C57BL/6 and NOD Lt/J backgrounds greater than 10 generations. IgH-deficient (13), STAT4-deficient (14), and STAT6-deficient (15) on the NOD background and CIITA-deficient mice (16) on the C57/BL6 background were purchased from Jackson ImmunoResearch Laboratories and bred to mice in our facility. All mice were housed in a pathogen-free barrier facility at University of California-San Francisco. Experiments complied with the Animal Welfare Act and National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were approved by the University of California-San Francisco Animal Care and Use Committee.

Antibodies
All Abs used for flow cytometry (anti-CD4 [RM4-5], CD8 [56-6.7], CD45 [30-F11], IL-4 [11B11], IL-10 [JES5-16E3], IL-17 [TC11-18H10], IFN-γ [XMG1.2] and isotype controls) were purchased from BD Biosciences. The anti-CD4 Ab GK1.5 and anti-CD8 Ab YTS-169.4 used for depletion experiments were gifts from Dr. Jeff Bluestone (University of California, San Francisco, CA).

Histology
Organs from mice were harvested, fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained for H&E. Tissue sections were scored on a grading system from 0 to 4, where 0 was no indication of immune infiltrate, 1 was a tissue that was 1–25% infiltrated, 2 was a tissue that was 26–50% infiltrated, 3 was a tissue that was 51–75% infiltrated, and 4 was a tissue that was greater than 76% infiltrated.

Immunostaining
Immune cell subtypes were visualized by immunohistochemistry using Abs specific for CD4, CD8, and IgD (BD Pharmingen) and a DAB staining kit (Vector Laboratories).

Adoptive transfer
Cervical lymph node cells (LNCs) and splenocytes were harvested and CD4+ or CD8+ T cells were depleted using complement. Cell populations (5 × 10^6 CD4+ and CD8+ depleted, or CD8+ depleted) were injected i.v. into SCID.NOD mice. On days 0, 5, 19, and 33 animals were treated with 0.5 mg/mouse of anti-CD4 (GK1.5, CD4+ depleted) or anti-CD8 (YTS169.4, CD8 depleted) injected i.p. to remove residual CD4+ or CD8+ T cells (17). Animals were aged 40 days post-transfer then sacrificed and analyzed as described.

Sera transfers
Wild-type or immunodeficient animals were injected i.p. with 150 microliters of wild-type or aire-deficient sera on day 0 followed by a repeat injection of 100 microliters on day 2. Animals were weighed three times a
week. Two weeks following the initial injection, animals were sacrificed and analyzed as described above. For repeat administration, wild-type animals were injected i.p. with 75 microliters of wild-type or aire-deficient sera every day, for 10 days (a total of 750 ul of sera). Animals were sacrificed and analyzed as described at day 14.

Flow cytometry
Salivary glands were incubated in 2 mg/ml collagenase D in DMEM supplemented with 10 μg DNase I for 40 min. The remaining tissue was dispersed by vortexing and filtered through nylon mesh. Cells were placed in culture in DMEM complete media with 10% FCS with Golgi-Stop (BD Biosciences) and stimulated with 10 ng/ml PMA and 0.5 μM ionomycin (Sigma-Aldrich) for 4 h at 37°C. After the incubation, cells were surface stained with Abs specific for CD4, CD8, and CD45 to gate lymphocytes, then permeabilized and stained with Abs specific for IL-4, IL-10, IL-17, IFN-γ, or isotype control. Cells were analyzed on a LSRII flow cytometer (BD Biosciences).

For LNC and splenic cell preparations, cervical lymph nodes or spleens were removed and dispersed by mechanical separation. Cells were then stimulated and analyzed as described above.

Anti-CD4 administration
Animals were given 500 micrograms of anti-CD4 (GK1.5) or rat isotype control (Jackson ImmunoResearch Laboratories) via i.p. injection once a week starting at age day 21. Animals were sacrificed on day 49. Histology was analyzed as described.

Statistics
Data was analyzed using Mann-Whitney nonparametric test with Prism software (GraphPad).
CD4⁺ and CD8⁺ T cells contribute to disease

Having confirmed that T cells are a critical cell type required for autoimmune pathogenesis, we sought to further delineate the role of CD4⁺ and CD8⁺ T cells in this process. To this end, we adoptively transferred aire-deficient lymphocyte populations depleted of CD4⁺ or CD8⁺ T cells into immunodeficient hosts. In this setting, aire-deficient lymphocytes devoid of CD4⁺ T cells had a diminished capacity to infiltrate target tissues. In fact, no evidence...
of immune infiltrates was observed in the lung, pancreas, reproductive organs (Fig. 3A), or eye (7). In addition, autoimmune infiltrates were reduced in the other organs analyzed – the lacrimal and salivary glands and the liver (Fig. 3A). Recipients of CD8+/H11001 T cell depleted lymphocyte populations, however, developed immune infiltrates that were no different from animals that received whole lymphocyte populations (Fig. 3A). These data suggested to us that CD4+/H11001 T cells are a key mediator of the autoimmune response in aire-deficient mice.

To further investigate the role of CD4+ T cells in the disease process, we bred aire-deficient mice to CIITA-deficient mice, which lack CIITA and peripheral class II expression, resulting in a near complete lack of mature CD4+/H11001 T cells (16). To confirm the critical role of CD4+/H11001 T cells in the pathogenesis of disease, we analyzed animals that were deficient for both aire and CIITA. In these mice, we found no evidence of autoimmunity against the eye or infiltrate into the retina (Fig. 3A). These data suggested to us that CD4+/H11001 T cells are a key mediator of the autoimmune response in aire-deficient mice.

During maturation, CD4+/H11001 T cells polarize into effector lineages including Th1-type, Th2-type, or Th17-type helper cells. To determine whether a specific path of development was being emphasized in the disease process in aire-deficient mice, we analyzed LNCs and organ-infiltrating lymphocytes for cytokine polarization. Lymphocytes from the cervical lymph node, spleen, and salivary gland were procured from aire-deficient C57BL/6 mice (n = 5) between 18 and 20 wk of age and cervical lymph node and spleen of aire-sufficient C57BL/6 mice (n = 5) that were age and gender matched. Cells were stimulated with PMA and ionomycin, and intracellular cytokine production was measured by flow cytometry. CD4+/H11001 and CD8+/H11001 T cells derived from wild type or aire-deficient spleens and cervical lymph nodes produced equivalent amounts of the cytokines IFN-γ, IL-4, IL-10, and IL-17 (Fig. 4). In contrast, T cells present in the autoimmune lesions of the salivary gland of aire-deficient mice produced dramatically increased levels in the infiltrated salivary and lacrimal glands (data not shown), a finding confirmed by flow cytometry of lymph nodes and spleen (data not shown). Thus, we hypothesize that the residual CD4+/H11001 T cells present in aire/CIITA DKO mice are capable of eliciting salivary and lacrimal gland autoimmunity, but are not of sufficient numbers to elicit autoimmunity against an immunoprivileged organ such as the eye.
of IFN-γ when stimulated with PMA and ionomycin (Fig. 4A). This increase in IFN-γ production was documented in both CD4+ and CD8+ subsets (Fig. 4, B and C). Expression of TNF-α was also observed in these cells (data not shown). We also observed a similar Th1 bias in the cells that infiltrate the lacrimal gland and retina (data not shown). In contrast, little expression of the Th2 cytokines IL-4 and IL-10 or the Th17 cytokine IL-17 was observed in cells derived from the autoimmune infiltrate. The data shown in Fig. 4 is representative of four independent experiments. Additionally, we believe that this polarization skewing was not due to the genetic background of the animals, as aire-deficient mice on the BALB/c and NOD background showed the same predisposition toward Th1 polarization (data not shown).

Genetic studies on the role of Th1 and Th2 polarization

To determine whether or not Th1 polarization was relevant to the disease process in aire-deficient mice, we generated mice deficient for both aire and either STAT4, a transcription factor required for potent Th1 polarization (14), or STAT6, a transcription factor required for Th2 polarization (15). To determine whether or not the disruption in STAT signaling resulted in a direct effect on the autoimmune response, tissue sections were prepared from the organs known to be infiltrated in age-matched aire-deficient, aire/STAT4 DKO and aire/STAT6 DKO mice. In the absence of STAT4 signaling, the degree of disease severity was decreased in the pancreas (Fig. 5, A and B), whereas in the absence of STAT6 signaling, the disease severity was increased in liver and salivary glands. We also performed a histological analysis on 20-wk-old aire/STAT4 DKO mice. Disease as measured by histological score was ultimately indistinguishable from aire-deficient animals. Thus, even in the absence of STAT4 signaling, the degree of disease severity was decreased in the pancreas (Fig. 5, A and B), whereas in the absence of STAT6 signaling, the disease severity was increased in liver and salivary glands.

Cellular therapies targeting CD4+ T cells

In light of the genetic evidence suggesting that CD4+ T cells are Th1 polarized and given the availability of anti-CD4 neutralizing Abs, we decided to investigate their potential as a therapeutic protocol in the modulation of disease. Administration of neutralizing anti-CD4 Ab has been shown to dramatically reduce the levels of circulating CD4+ T cells in the blood and lymph nodes (25). In addition, depleting CD4 has shown efficacy in other T cell-mediated autoimmune models (26). We injected aire-deficient NOD mice with a regimen of 500 micrograms of anti-CD4 Ab (GK1.5) or isotype control once a week, starting at 3 wk of age. Administration of anti-CD4 with our protocol resulted in a significant depletion of CD4+ T cells in treated animals (data not shown). This...
treatment protocol also significantly ameliorated immune infiltration in multiple organs. The histological slides were scored as described and demonstrated a significant decrease in immune infiltrate in the retina, liver, lung, and reproductive organs (Fig. 6A). Histology revealed dense mononuclear infiltrates in the retina and lung along with a loss of normal tissue architecture in both tissues, but no obvious immune infiltrate or defects in anti-CD4-treated mice (Fig. 6B).

**Discussion**

APS1 is known to be a monogenic, recessive autoimmune disease that primarily affects organs of the endocrine axis. Due to the complications of studying disease in humans, little is known about the pathogenicity of B and T cells in APS1. Although many previous reports have focused on the presence of autoantibodies in both aire-deficient animals and APS1 patients, no reports have demonstrated a pathogenic role for either B or T cells. In this study, we show that T cells are absolutely critical to autoimmunity by generating mice deficient for both aire and TCRα. These mice, which lack αβ T cells, are completely healthy and free of immune infiltrates. To further delineate the effective contribution of CD4+ and CD8+ T cells to this process, we performed adoptive transfer of lymphocyte populations depleted of CD4+ or CD8+ T cells and analyzed CIA-Deficient mice. In both cases, CD4+ T cells appeared to play a major role in disease pathogenesis. Upon further dissection, many of the CD4+ T cells resident in the infiltrated tissues produced the Th1 cytokine IFN-γ while few produced IL-17 or IL-10. The importance of Th1-like cells being effectors in aire-deficient mice was confirmed by genetic studies using STAT4- and STAT6-deficient animals. Due to these findings, we reasoned that depletion of CD4+ T cells in vivo would be an effective means of modulating disease. Indeed, administration of neutralizing Abs significantly altered the degree of infiltration in tissues targeted by the immune system.

Although the number of case reports described in the primary literature is limited, published studies of immunosuppressive regimens in APS1 patients suggest synergy with our data. In a patient of French-Canadian descent, several aspects of the disease responded to cyclosporine A treatment, including a remission of exocrine pancreatitis, keratoconjunctivitis, and alopecia (27). A 5-year-old patient of Iranian descent who underwent two liver transplants responded poorly to the immunosuppressive regimen initiated during the first transplant, but well to the regimen initiated during the second transplant. In the former, prednisone, azathioprine, and cyclosporine A was administered; however, autoantibodies were detected to multiple known organ targets and disease progression was unchecked. In the latter case, the patient was given tacrolimus, prednisone, and mycophenolate mofetil and all APS1-related symptoms (including candidiasis and autoantibodies) decreased (28). Finally, in a study on children with autoimmune hepatitis and APS1, two of three patients responded well to steroids and azathioprine (29). Our own results in the mouse model would suggest that T cell targeted therapies may hold the best promise, as specific depletion of CD4+ T cells significantly ameliorates disease.

One question unanswered by the human data is the relative role of B cells in disease pathogenesis, as the immunosuppressive regimen administered equally affect B and T lymphocytes. Despite the identification of many autoantibody specificities in both the aire-deficient mouse and APS1 patients, our data suggests that B cells play a limited role in disease pathogenesis in the mouse model. Sera transfers of autoantibodies were unable to elicit any observable autoimmunity, and a genetic deficiency in the B cell compartment had a limited effect on the autoimmune infiltrates.

Why then is the generation of multiple autoantigen specificities observed so clearly? The loss of central tolerance in the absence of Aire permits the escape of high-affinity autoreactive T cells (20, 21). It is likely that these T cells, upon their escape into the periphery, provide B cell help and result in the generation of high affinity autoantibodies. It remains possible, however, that B cells influence additional aspects of the disease not analyzed in this work. For example, APS1 patients have been reported whose clinical history include autoimmune diseases such as Graves and idiopathic thrombocytopenic purpura (1), in which a clear role for a pathogenic autoantibody has been established. To date, no one has established such a disease mechanism in the mouse model.

Recently, there has been a vigorous discussion in the literature about the potential parallels between this animal model and human patients (30–32). Our findings in the animal model, when compared with the extensive clinical experience of clinicians (1, 33), indicate that a majority of the organs targeted in the absence of Aire expression are shared between the two species (1, 34). Thus, aire-deficient mice and APS1 patients have been reported to develop adrenal failure, hypoparathyroidism, premature ovarian failure, thyroiditis, autoimmune hepatitis, exocrine pancreatitis, gastritis, pneumonia, dacryoadenitis, and sialitis. Further strengthening the connection between the mouse model and APS1 is preliminary data in our lab where we have found an APS1 patient with photoreceptor-specific autoantibodies and retinitis that is highly similar to our published work (7) detailing the uveitis in aire-deficient mice (unpublished data).

The larger view of what drives autoimmunity in the absence of Aire is becoming increasingly clear. Previous results from our lab identified IRBP as the primary target of the immune response in the retina. Confirming an important role of aire in autoantigen expression within mTECs, transplantation of a thymus derived from an IRBP-deficient mouse into nude recipients was sufficient to result in autoimmunity (7). Thus, absence of a single Ag within the thymic compartment, despite the presence of functional aire protein, allowed for the generation of an aberrant immune response. This immune response likely results from a failure in negative selection, rather than a deficit in positive selection of regulatory T cells. In the murine model, no defect in the number or selection of FoxP3+ regulatory T cells has been reported (20), although data in humans remains controversial (35). Recent data also suggests that the innate immune response, in particular signals generated through TLRs, plays a limited role in driving the autoimmunity in the mouse model (36).

In what direction, then, does the future of APS1 treatment lie? Our results suggest that specific targeting of T cells may be an effective way to modulate disease. Although depletion of CD4+ T cells in APS1 patients may not be a practical clinical approach given the severe immunosuppression associated with it, other Ab-based therapies may prove useful. For example, immunomodulation using mAbs against CD3 has been proven to delay onset of the T cell-driven disease, type 1 Diabetes and also is associated with more acceptable levels of immunosuppression (37). Likewise, targeting of Th1-related cytokines such as IFN-γ or TNF-α may prove to be efficacious given our data supporting a role for Th1 T cells as key effectors in the mouse model. Finally, identifying the autoantigens that are targeted in APS1 patients could result in Ag-specific therapies involving the suppression of activated autoreactive cells, via regulatory T cells, or other forms of Ag-specific tolerance such as coupled cell tolerance (38, 39). In support of this notion is recent data from our group that demonstrated that the uveitis in the mouse model is driven by a single self-Ag (7). The
studies presented here should be used to help provide the framework for tar-
gen immunotherapy that can be used in the treatment of this rare, but severe, clinical syndrome.

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Disclosures
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