Aire Deficiency Promotes TRP-1–Specific Immune Rejection of Melanoma

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Abstract

The thymic transcription factor autoimmune regulator (Aire) prevents autoimmunity in part by promoting expression of tissue-specific self-antigens, which include many cancer antigens. For example, AIRE-deficient patients are predisposed to vitiligo, an autoimmune disease of melanocytes that is often triggered by efficacious immunotherapies against melanoma. Therefore, we hypothesized that Aire deficiency in mice may elevate immune responses to cancer and provide insights into how such responses might be triggered. In this study, we show that Aire deficiency decreases thymic expression of TRP-1 (TYRP1), which is a self-antigen in melanocytes and a cancer antigen in melanomas. Aire deficiency resulted in defective negative selection of TRP-1–specific T cells without affecting thymic numbers of regulatory T cells. Aire-deficient mice displayed elevated T-cell immune responses that were associated with suppression of melanoma outgrowth. Furthermore, transplantation of Aire-deficient thymic stroma was sufficient to confer more effective immune rejection of melanoma in an otherwise Aire wild-type host. Together, our work showed how Aire deficiency can enhance immune responses against melanoma and how manipulating TRP-1–specific T-cell negative selection may offer a logical strategy to enhance immune rejection of melanoma. Cancer Res; 1–13. ©2013 AACR.

Introduction

Melanoma is the deadliest form of skin cancer and accounts for 5% of cancer deaths in the United States (1). Understanding mechanisms that may limit melanoma development is therefore a significant public health concern. The cellular immune response has the potential to eradicate melanoma cells, and spontaneous regression of melanoma associated with lymphocytic infiltration of tumors has been reported (2, 3). Despite this potential, the immune response is often ineffective, with high rates of melanoma growth occurring in immunocompetent individuals. Furthermore, while immunotherapy for metastatic melanoma can result in clinical benefit, a complete response occurs in only a small proportion of patients (4). Overcoming immune tolerance to melanoma in otherwise healthy individuals may therefore be important for preventing and treating melanoma.

Several antigens targeted by the immune response in melanoma are also antigens in vitiligo, an acquired depigmenting disorder resulting from autoimmune destruction of melanocytes (5–7). Consistent with shared antigens in these 2 immune settings, the development of melanoma is regularly accompanied by vitiligo in a number of animal models (6). In addition, development of vitiligo in patients with advanced melanoma undergoing immunotherapy is associated with improved prognosis (8). This overlap in immune responses to vitiligo and melanoma suggests that factors that result in vitiligo may also enhance the immune response against melanoma.

The autoimmune regulator (Aire) gene promotes organ-specific immune tolerance and prevents the development of a number of autoimmune diseases including vitiligo. Patients with mutations in Aire have an 18- to 36-fold increased risk of developing vitiligo compared with the general population (9), and genetic polymorphisms in Aire have been associated with vitiligo development in a case–control study (10). Within the thymus, Aire upregulates expression of a large number of tissue-specific self-antigens in medullary thymic epithelial cells (mTEC; refs. 11–13). Ectopic expression of these self-antigens drives the clonal deletion of developing T cells recognizing these antigens with high affinity (14, 15). In addition, Aire may have other functions affecting T-cell tolerance including possible roles in antigen processing/presentation and the development of regulatory T cells (Tregs; refs. 14, 16, 17).

The existence of shared vitiligo/melanoma antigens suggests that decreased Aire function in the thymus, in addition to predisposing to vitiligo, may also enhance immune rejection of melanoma. In support of this hypothesis, Aire polymorphisms have been associated with melanoma development in a small case–control study (18). In addition, Aire-deficient mice may have increased memory response to melanoma following immunization with irradiated melanoma cells (19). At the same time, however, recent work also suggests that thymic expression of a cancer-germline antigen only minimally

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changes T-cell responses to antigen-expressing tumors (20). In addition, Aire-regulated thymic expression of tyrosinase, a shared vitiligo/melanoma antigen, does not shape the tyrosinase-specific T-cell repertoire (21). Instead, the tyrosinase-specific T-cell repertoire depends on constitutive presentation of tyrosinase in peripheral lymphoid organs. Thus, although there is some evidence that thymic Aire function regulates the immune response to melanoma, further studies are needed to clarify this issue. Moreover, the mechanism by which Aire may regulate the immune response to melanoma has not been delineated.

We show here that Aire normally induces thymic expression of the shared vitiligo/melanoma antigen, TRP-1 (tyrosinase related protein-1; Tyrp1; gp75), and lack of thymic TRP-1 expression in Aire-deficient mice results in the escape of TRP-1-specific T cells from clonal deletion. Aire-deficient mice generate a more activated primary T-cell response to melanoma that is associated with delayed growth of melanoma. In addition, transplantation of thymic stroma is sufficient to confer more effective immune rejection of melanoma. Together, these findings establish that Aire deficiency enhances the primary T-cell response to melanoma and delineate a novel pathway in which Aire deficiency promotes an antimelanoma T-cell response by decreased TRP-1 expression in the thymus.

Materials and Methods

Mice

Tyrp1bw TRP-1-specific T-cell receptor (TCR) transgenic mouse model (TRP-1 TCR Tg) RAG–/– mice (JAX stock number 008684) were purchased from The Jackson Laboratory. B6. AireGW/þ mice were backcrossed more than 10 generations from a mixed C57BL/6-129 line described in ref. (22). To generate TRP-1–specific CD4þ TCR Tg mice in an Aire-deficient background, male Tyrp1bw TRP-1 Tg RAG–/– mice were crossed with female B6. AireGW/þ mice. F1 male mice with genotype B6. AireGW/þ Tyrp1bw/þ TRP-1 TCR Tg RAG–/– mice were crossed with female B6. AireGW/þ mice. F1 male mice with genotype B6. AireGW/þ Tyrp1bw/þ TRP-1 TCR Tg RAG–/– were further crossed with male CD4–/– mice. F2 mice with genotype B6. AireGW/þ Tyrp1bw/þ TRP-1 TCR Tg RAG–/– and B6. AireGW/þ Tyrp1bw/þ TRP-1 TCR Tg RAG–/– were used for tumor inoculation studies. B6.Nude mice were purchased from Jackson Laboratory. All mice were used in accordance with guidelines from the University of North Carolina, Chapel Hill Animal Care and Use Committee. All experiments were performed on March 6, 2021. © 2013 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on March 6, 2021. © 2013 American Association for Cancer Research.

Antibodies and flow cytometry

Anti-CD4 (RM4-5), anti-CD3 (145-2c11), anti-CD25 (PC61), anti-CD69 (IM7), anti-CD62L (MEL-14), and anti-CD44 (IM7) were purchased from BD Biosciences. Intra- cellular staining for cytokines was carried out with the Cytofix/ Cytoperm Intracellular Staining Kit (BD Biosciences) as per manufacturer’s instructions. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 5 hours before antibody staining. FOXP3 Staining Buffer Set was used for FOXP3 intracellular staining according to manufacturer’s instructions (eBioscience). All samples were run on a Dako CyAn (Beckman-Coulter) flow cytometer.

B16 melanoma model

B16-F10 is a TRP-1þ C57BL/6-derived melanoma cell line that was kindly provided by Dr. Alan Fong (University of North Carolina, Chapel Hill). It was originally from American Type Culture Collection and maintained in culture media as previously described (23, 24). Mice were injected subcutaneously with 1 × 105 B16 melanoma cells in 100 μL volume. Perpendicular tumor diameters were measured with calipers. Body weight and physiologic status was monitored daily.

Real-time RT-PCR

Thymic stromal preparations and real-time reverse transcriptase PCR (RT-PCR) were conducted as previously described (25). TaqMan primer/probe sets for TRP-1, tyrosinase, and silver/gp100 were purchased from Applied Biosystems.

Bone marrow chimeras and thymic transplants

Bone marrow chimeras and thymic transplants were conducted as previously described (22). For bone marrow chimeras, bone marrow was harvested from the femurs of 6-week-old Tyrp1bw TRP-1 Tg RAG–/– mice. T cells were removed from the bone marrow by complement depletion using antibodies against CD4 and CD8. Recipient mice received 2 doses of radiation (500 rad each time) at least 4 hours apart. Cells (1 × 107) were injected retro-orbitally into each recipient mouse. Chimeras were aged for 10 weeks before analysis.

For thymic transplants, thymi from neonates were removed and cultured in Transwell plates for 7 days in L35 mmol/L 2’-deoxyguanosine (2-dG; Sigma-Aldrich) in complete Dulbecco’s modified Eagle’s medium (DMEM) to deplete hematopoietic cells. Thymi were washed in complete DMEM (without 2-dG) 2 hours before transplantation. Thymi were transplanted under the kidney capsule of B6.Nude recipients. Transplanted mice were aged for 10 weeks before analysis. T-cell reconstitution was confirmed by the presence of CD3+, CD4+, and CD8+ cells in the peripheral blood.

ELISA

B16 melanoma cell extract or TRP-1109–130 peptide (NCGTCRPGWWRGACNQKILTRGL) was incubated overnight at 4°C in 96-well ELISA microwell plates (Xenopore) according to the manufacturer’s instructions. After blocking with 3% bovine serum albumin in PBS, mouse serum was added at a 1:200 dilution in PBS and incubated for 2 hours at room temperature. Goat anti-mouse immunoglobulin G (IgG; γ-chain specific) horseradish peroxidase–conjugate were used to detect antibodies, respectively. ELISA was developed and read using a Molecular Device ELISA Reader and analyzed with the instrument’s software. Relevant positive controls (anti-TRP-1 antibody) and negative controls (no serum, antihuman IgG alone) were conducted in parallel. Assays were conducted at least twice with wells plated in 5 replicates for verification of results.

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Immunofluorescence staining

Tumors from mice were harvested and fixed overnight in 10% formalin at 4 °C, then stored in 70% ethanol. Tumors were embedded in paraffin for sectioning. Slides were stained over-night with anti-CD3 antibody, washed, and mounted with 4’,6-diamidino-2-phenylindole (DAPI). Images were taken on a fluorescence microscope (Olympus BX60) and analyzed with ImageJ software.

Isolation of tumor-infiltrating lymphocytes

Tumors were dissected and minced before incubation with collagenase type I/IV, hyaluronidase, and DNase I. T cells were isolated using immunomagnetic bead separation using type MS- or VS+ columns and anti-CD3–conjugated magnetic beads (Miltenyi) according to the manufacturer’s instructions.

Adoptive transfer

Spleens from Tyrpl+/−TRP-1 TCR Tg RAG−/− male mice were harvested and made into single-cell suspensions. Red blood cells were eliminated by ammonium-chloride-potassium lysis. Subsequently, cells were counted and enriched for CD4+ CD25+ and CD4+CD25− T cells by magnetic bead sorting using a CD4+CD25+ T-cell enrichment kit from Miltenyi Biotec. CD4+CD25+ and CD4+CD25− T cells were resuspended in PBS and injected retro-orbitally into B6.RAG−/− recipient mice.

Statistical analysis

PRISM 5.0 and Microsoft Office Excel software was used to analyze data (GraphPad Software, Inc.). Unpaired Student t tests were used to compare the differences between 2 groups. Survival curves were compared by Mann–Whitney U test. P values of 0.05 or less were considered significant.

Results

Aire deficiency is associated with decreased growth of B16 melanoma cells and increased density of tumor infiltrating lymphocytes

Mice with Aire deficiency do not spontaneously develop clinical vitiligo but may have features of the autoimmune vitiligo response. A feature of the autoimmune response in vitiligo is the development of autoantibodies against melanocyte antigens (26). We sought to determine if Aire-deficient mice harbor autoantibodies against melanocyte antigens by measuring the frequency of serum autoantibodies against B16 melanoma cell extract. As a model of Aire deficiency, we used a C57/BL6 mouse line harboring a dominant Aire G228W point mutation that results in hypomorphic Aire function [B6.AireGW/+ mice; (22)]. Increased serum autoantibodies against melanoma cell extract were seen in 15- to 25-week-old B6.AireGW/+ mice compared with wild-type (B6.Aire+/+) littermate controls (Supplementary Fig. S1A, left). Consistent with these findings, increased serum autoantibodies against melanoma cell extract were also seen in 15- to 25-week-old Aire knockout (B6.Aire−/−) mice (Supplementary Fig. S1A, right). These findings suggest that Aire deficiency promotes an autoimmune response against melanocyte antigens.

Because Aire-deficient mice show increased immune response to B16 cell extract, we hypothesized that Aire-deficient mice would eradicate B16 melanoma cells more effectively. To test this hypothesis in vivo, we inoculated B16 melanoma cells subcutaneously on the flanks of B6.AireGW/+ mice and followed tumor growth. B16 melanoma cells grew more slowly in B6.AireGW/+ mice compared with B6.Aire+/+ littermate controls (Fig. 1A and B and Supplementary Fig. S1B). Between day 16 and 19, the average size of tumors was significantly reduced in B6.AireGW/+ compared with B6.Aire+/+ littermate controls (Fig. 1B). Consistent with a role for Aire in mediating B16 melanoma growth, B16 melanoma cells inoculated into B6.Aire−/− mice also grew more slowly compared with B6.Aire+/+ mice (Fig. 1B). In addition, improved survival was seen in Aire-deficient B6.AireGW/+ and B6.Aire−/− mice inoculated with B16 melanoma cells compared with B6.Aire+/+ littermate controls (Fig. IC).

The decreased B16 melanoma growth in Aire-deficient mice was associated with increased T-cell immune infiltration within the tumors. We used immunofluorescence staining for CD3 to quantitate the number of infiltrating T cells within the tumor. An increased density of CD3+ T cells per tumor area were found in the tumors of B6.AireGW/+ mice compared with B6.Aire+/+ mice (Fig. 1D and E). Increased numbers of tumor-infiltrating lymphocytes in the B16 melanoma per gram of tumor in Aire-deficient mice was confirmed by flow-cytometric analysis of the tumors (data not shown). This finding suggests that an increased T-cell response directed at melanoma is associated with decreased tumor growth in Aire-deficient mice.

Aire deficiency is associated with activated immune response against melanoma

We sought to determine whether tumor-infiltrating T cells were not only more numerous but also more activated in B6. AireGW/+ mice. We pooled together lymphocytes from the tumors of 6 B6.AireGW/+ and 6 wild-type littermate controls to collect a sufficient number of events to analyze. An increased frequency CD4+ and CD8+ T lymphocytes with an activated/memory phenotype (CD62Llow CD44high) were found within melanoma of B6.AireGW/+ mice compared with wild-type littermates (Fig. 2A). An increased frequency of CD62Llow CD44high CD4+ and CD8+ T cells were also seen in the spleen and tumor-draining lymph node (Supplementary Fig. S2).

We next assessed the ability of tumor-infiltrating lymphocytes to secrete proinflammatory cytokines by intracellular cytokine staining. We evaluated the ability of T cells to secrete interleukin (IL)-2 and IFN-γ, as both cytokines have been implicated in rejection of tumors by T cells (27–29). An increased frequency of IL-2 and IFN-γ–secreting CD4+ and CD8+ tumor-infiltrating lymphocytes were detected in B6. AireGW/+ mice compared with wild-type littermate controls (Fig. 2B and C). An increased frequency of CD4+ and CD8+ tumor-infiltrating lymphocytes secreting IL-2 and IFN-γ was also seen in spleen and tumor-draining lymph nodes of B6.AireGW/+ mice compared with wild-type littermates (Fig. 2D and E). Importantly, we did not observe a difference in the frequency of memory/activated or cytokine-secreting T cells in B6.AireGW/+ mice before tumor inoculation (Supplementary Fig. S3A–S3D). The increased immune activation in B6.AireGW/+ mice therefore, is
specific to the antitumor response and does not represent nonspecific immune activation. Taken together, these data suggest that melanoma provokes an enhanced T-cell response in Aire-deficient mice.

**Aire deficiency in thymic stroma is sufficient to augment immune response against melanoma**

Although Aire is most highly expressed in the thymus, peripheral Aire expression in the lymph node and spleen also promotes clonal deletion of autoreactive T cells (30). We sought to determine whether central (thymic) Aire expression was sufficient to limit the immune response toward melanoma. To isolate Aire deficiency to the thymus, we transplanted thymic stroma from B6.AireGW/+ mice or wild-type littermates into athymic nude (Foxn1-/-; B6.Nude) mice. The reconstituted mice have wild-type Aire expression in the periphery but are either Aire-deficient or Aire-wild-type in the thymus (Fig. 3A). Thymic engraftment and immune reconstitution was determined by monitoring for the presence of CD3+, CD4+, and CD8- T cells in the peripheral blood 8 weeks posttransplantation. No significant difference in CD4+ and CD8- T-cell reconstitution was found between recipients of B6.AireGW/+ or B6.Aire-/- thymi (Supplementary Fig. S4).

After confirming immune reconstitution in transplanted mice, we inoculated B16 melanoma cells and monitored tumor growth (Fig. 3A). Between days 9 and 19 postinoculation, melanoma growth was significantly decreased in mice reconstituted with Aire-deficient thymi compared with wild-type thymi (Fig. 3B). This decreased growth was associated with increased numbers of CD3+ tumor-infiltrating lymphocytes per area of tumor (mm²; Fig. 3C and D). In addition, an increased frequency of IL-2 and IFN-γ-secreting CD4+ and CD8+ tumor infiltrating lymphocytes were seen in mice reconstituted with Aire-deficient thymi (Fig. 3E and F). An increased frequency of IL-2 and IFN-γ-secreting CD4+ and CD8+ splenocytes was also observed in mice reconstituted with Aire-deficient thymi (Fig. 3G and H). An enhanced antitumor immune response therefore tracks with Aire-deficient thymic stroma.

**Defective negative selection of TRP-1–specific T cells in Aire-deficient thymus**

To define the mechanism by which thymic Aire limits the immune response against melanoma, we sought to determine which antigens targeted in both vitiligo and melanoma might be Aire-regulated in the thymus. mTECs from B6.AireGW/+ mice and B6.Aire-/- mice were isolated and relative expression levels of 3 shared vitiligo/melanoma antigens (TRP-1, tyrosinase, and gp100) were determined by real-time RT-PCR. While expression of gp100 was independent of Aire, expression...
Aire deficiency enhances immune response to TRP-1

Figure 2. Aire-deficient mice inoculated with B16 melanoma have activated T cells with increased proinflammatory cytokine production. A, flow cytometry plots of CD62L and CD44 expression in tumor-infiltrating lymphocytes from AireGW/− or wild-type (WT) mice at day 18. B and C, flow cytometry plots of intracellular expression of IL-2 (B) and IFN-γ (C) in tumor-infiltrating lymphocytes. D and E, flow cytometry plots of intracellular expression of IL-2 (D) and IFN-γ (E) in either lymphocytes of spleen and tumor-draining lymph node (LN). Cumulative data are shown in the right. n = 4 for both groups. Data shown are representative experiment of 3 experiments. *P < 0.05, error bars represent SEM.

of TRP-1 and tyrosinase was significantly decreased in B6. AireGW/− thymi (Fig. 4A). Thus, Aire-mediated thymic expression of either TRP-1 or tyrosinase could potentially mediate T-cell tolerance toward melanoma.

We chose to focus on Aire-mediated thymic expression of TRP-1 rather than tyrosinase, because thymic tyrosinase expression has previously been shown to have no effect on the immune response against melanoma (21). Importantly, TRP-1 is expressed in B16 melanoma cells (31), and is therefore a potential antigenic target in immune rejection of B16 melanoma cells. Furthermore, both B6.Aire−/− and B6.AireGW/− mice have increased serum autoantibodies against TRP-1, suggesting a break in self-tolerance toward this antigen in Aire-deficient mice (Fig. 4B).
Figure 3. Augmented response against melanoma maps to Aire differency in thymic stroma. A, scheme for thymic transplantation experiment. Thymic stroma from Aire^{GW/+} mice or wild-type (WT) mice were transplanted under the kidney capsule of athymic C57/BL6 Foxn1^{-/-} (B6.Nude) mice. After 2 months to allow T-cell reconstitution, mice were injected with B16 melanoma cells subcutaneously and tumor growth was monitored. B, tumor growth in B6.Nude recipient mice reconstituted with Aire^{GW/+} or wild-type thymic stroma. n = 4 for both groups. C, representative immunofluorescence staining of CD3 (red) and DAPI for tumor nuclei (blue) on tumor sections from mice reconstituted with Aire^{GW/+} or wild-type thymus. Four tumor sections were evaluated for each group and 6 areas from each section were randomly selected. D, average densities of CD3+ T cells per tumor area (mm^2) in tumors of mice reconstituted with Aire^{GW/+} or wild-type thymic stroma. E and F, tumor-infiltrating lymphocytes were isolated from mice reconstituted with Aire^{GW/+} or wild-type thymus, and intracellular expression of IL-2 (E) and IFN-γ (F) was evaluated by flow cytometry. G and H, intracellular expression of IL-2 (G) and IFN-γ (H) from either CD4+ or CD8+ lymphocytes in spleen was evaluated by flow cytometry. Cumulative data are shown in right. n = 4 for both groups. * P < 0.05, error bars represent SEM.

We used an MHC class II-restricted TRP-1 TCR Tg to track TRP-1–specific T cells within the thymus (31). The TRP-1 TCR Tg mouse line is derived from a CD4+ T cell cloned from a TRP-1–deficient mouse (Tyrp1^{B-w}) immunized with TRP-1 protein. TRP-1 TCR Tg mice were crossed onto a recombinase-activating gene (RAG)–deficient (RAG^{−/−}) background to prevent endogenous TCR rearrangement. Adoptive transfer of naïve TRP-1 TCR Tg RAG^{−/−} cells are capable of eradicating established B16 melanoma tumors in a lymphopenic host (32). Thus, these TRP-1–specific CD4+ T cells are directly relevant in the immune response against B16 melanoma.
In thymi deficient for the TRP-1 antigen (Tyrp1<sup>B-w</sup>), approximately 15% of lymphocyte-gated cells were single positive for CD4 (Fig. 4C, left). In thymi in which cognate antigen is expressed (Tyrp1<sup>+/-</sup>), on the other hand, the frequency of CD4 single positive cells is significantly decreased (1%–2%; Fig. 4C, middle), indicating efficient negative selection of this population. This change was also seen with the frequency of clonotype-specific Vβ14<sup>+</sup> CD4 single positive cells among total thymocytes (Fig. 4D). In addition to decreased frequency of CD4 single positive cells, the absolute number of CD4 single positive thymocytes was also significantly decreased [9 × 10<sup>5</sup> cells in Tyrp1<sup>B-w</sup> thymi compared with 3 × 10<sup>6</sup> cells in Tyrp1<sup>+/-</sup> thymi (Fig. 4E)]. Thus, consistent with a previous report (31), effective negative selection of TRP-1 TCR Tg RAG<sup>−/−</sup> cells occurs in thymi expressing normal levels of TRP-1 antigen.

Because TRP-1 expression is decreased in Aire-deficient thymi (Fig. 4A), we reasoned that Aire deficiency would result in defective negative selection of TRP-1 TCR Tg RAG<sup>−/−</sup> cells. We therefore generated TRP-1 TCR Tg RAG<sup>−/−</sup> mice on an Aire-deficient background (B6.Aire<sup>GW</sup>/AIRE<sup>−/−</sup> TRP-1 TCR Tg RAG<sup>−/−</sup> mice) and characterized T-cell development within the thymi of these mice. The frequency of CD4 single positive cells was significantly greater in Aire-deficient thymi compared with thymi expressing normal levels of TRP-1 antigen (Fig. 4C, compare middle and right). Similar trends were also seen when the frequency of clonotype-specific Vβ14<sup>+</sup> CD4 single positive T cells were assessed as a percentage of total thymocytes (Fig. 4D). The absolute number of CD4 single positive cells was also significantly greater in Aire-deficient thymi (1 × 10<sup>6</sup>) compared with thymi expressing normal levels of TRP-1 antigen (3 × 10<sup>5</sup> cells; Fig. 4E). Splenic CD4<sup>+</sup> T cells reflected the findings in the thymus (Fig. 4F–H).

To investigate thymic negative selection using an alternative approach, we carried out bone marrow chimera experiments in which Tyrp1<sup>B-w</sup> TRP-1 TCR Tg RAG<sup>−/−</sup> bone marrow was transplanted into lethally irradiated B6.Aire<sup>GW</sup>/+ or B6.Aire<sup>−/−</sup> recipients (Supplementary Fig. S5). Within these chimeric
mice, TRP-1-specific T cells would develop in either an Aire-deficient or Aire wild-type thymic stromal environment. Increased frequency of CD4 single positive cells was seen in Aire-deficient recipient thymi (~25%) compared with wild-type recipient thymi (~4%). Consistent with this, an increased frequency of clonotype-specific T cells was also seen in Aire-deficient thymus. In this bone marrow chimera model, then, Aire-deficient thymic stroma also results in defective negative selection of TRP-1-specific CD4+ T cells.

**Numbers of TRP-1-specific Tregs are unchanged in the thymi of Aire-deficient mice**

The TRP-1 TCR Tg mouse line is unusual in that CD4+ FOXP3+ T reg cells in the thymus even in the RAG–/– setting (32). To investigate the effect of Aire in the thymic selection of TRP-1-specific Tregs within the thymus, we compared the numbers of TRP-1-specific Tregs in thymi of B6. AireGW/+ and B6.Aire–/– mice. Although the percentage of FOXP3+ Tregs among CD4+ single positive thymocytes is decreased in B6.AireGW/+ TRP-1 TCR Tg RAG–/– thymi compared with B6.Aire–/– TRP-1 TCR Tg RAG–/– mice (Fig. 5A and B), the absolute numbers of these cells was unchanged (Fig. 5C). Interestingly, a greater proportion of CD4+ T cells expressing an intermediate level of FOXP3 were seen in thymi of Aire-deficient versus Aire wild-type TRP-1 TCR Tg Rag–/– thymi (Fig. 5A and D). In addition, the frequency of FOXP3+ Tregs is also decreased in the spleen of Aire-deficient mice but the absolute numbers are increased (Supplementary Fig. S6A–S6C). Similar to the thymus, a greater proportion of CD4+ T cells expressing an intermediate level of FOXP3 were seen in the spleen of Aire-deficient mice (Supplementary Fig. S6A and S6D).

Because Aire deficiency results in defective negative selection of TRP-1-specific CD4+ T cells without affecting CD4+ Treg numbers, we sought to determine the ratio of CD4+ effectors (defined as CD4+ FOXP3−) to CD4+ Tregs (CD4+ FOXP3+) in both the thymus and the periphery. The effector: Treg ratio was higher in both thymus and spleen of Aire-deficient mice (Fig. 5D). This increased ratio suggests that the balance of effector:Tregs is tipped toward increased effectors in Aire-deficient mice.

**Decreased B16 melanoma growth and enhanced T-cell response in Aire-deficient TRP-1 TCR Tg mice**

We tested whether the greater proportion of TRP-1-specific T effectors in Aire-deficient mice might be linked with more effective eradication of melanoma in these mice. To do this, we inoculated B16 melanoma cells into either B6.AireGW/+ TRP-1

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**Figure 5.** Aire does not affect numbers of TRP-1-specific CD4+ FOXP3+ Tregs in the thymus. A, representative flow cytometry plots of CD4 and FOXP3 expression in CD4+ thymocytes from AireGW/+ TRP-1 TCR Tg RAG–/– or Aire–/– TRP-1 TCR Tg RAG–/– mice. B and C, cumulative data for percentage of CD4+ FOXP3+ cells among total CD4 single positive thymocytes (B) and absolute number of CD4+ FOXP3+ cells (C) in thymi are shown. Each shape represents an individual mouse. D, cumulative data for percentage of CD4+ FOXP3 intermediate (int) and CD4+ FOXP3 high (hi) cells among total CD4 single positive thymocytes. E, cumulative data of effector (CD4+ FOXP3−) versus Treg (CD4+ FOXP3+) ratios in thymocytes (left) and splenocytes (right) from AireGW/+ or Aire–/– TRP-1 TCR Tg RAG–/– mice.
TCR Tg RAG−/− or B6.Aire+/+ TRP-1 TCR Tg RAG−/− mice and monitored tumor growth. Growth of B16 melanoma tumor was significantly decreased in Aire-deficient mice compared with Aire-wild-type controls between days 19 and 26 (Fig. 6A and B). In addition, Aire-deficient mice inoculated with B16 melanoma showed significantly increased survival compared with Aire-wild-type controls (Fig. 6C).

Decreased B16 melanoma growth was associated with increased density of tumor-infiltrating T cells in B6.Aire+/+ TRP-1 TCR Tg RAG−/− mice. On immunofluorescent staining of tumor sections, the density of CD3+ T cells was higher in tumors from Aire-deficient mice compared with Aire-wild-type controls (Fig. 6D and E). This increased density of tumor-infiltrating lymphocytes was associated with increased absolute numbers of activated/memory (CD62Llow CD44high) TRP-1–specific CD4+ T cells in the spleen and tumor-draining lymph nodes (Fig. 6F). In addition, increased absolute numbers of IL-2 and IFN-γ secreting TRP-1–specific CD4+ T cells in the spleen and tumor-draining lymph nodes were also seen (Fig. 6G).

Thus, enhanced melanoma rejection in Aire-deficient TRP-1 TCR Tg mice is associated with both increased density of tumor-infiltrating lymphocytes and increased numbers of activated cells producing proinflammatory cytokines.

As shown in Fig. 5E, the ratio of effector:Tregs is increased in Aire-deficient TRP-1 TCR Tg RAG−/− mice. We therefore sought to determine whether this increased ratio in Aire-deficient mice might contribute to increased melanoma growth suppression. To test this, we conducted adoptive transfers of T cells into RAG-deficient recipients at the 2 effector to Treg ratios seen in wild-type (effector:regulatory 1:3) and Aire-deficient (effector:regulatory 2:1) mice. Adoptive transfer of T cells at the effector to Treg ratio in spleen of Aire-deficient mice protected against melanoma outgrowth, whereas adoptive transfer of T cells at the effector to Treg ratio in spleen of wild-type mice did not (Fig. 6H).

These findings suggest that the increased proportion of Tregs may prevent efficient antimalanoma immunity in Aire wild-type mice.

Discussion

This study defines a novel pathway by which Aire deficiency enhances T-cell–mediated immune rejection of melanoma. In wild-type mice, Aire-mediated expression of the shared vitiligo/melanoma antigen TRP-1 in thymic mTECs promotes clonal deletion of TRP-1–specific CD4+ T cells and immune tolerance toward melanoma (Fig. 7A). In Aire-deficient mice, on the other hand, lack of Aire-driven TRP-1 expression in thymic mTECs results in defective negative selection of TRP-1–specific T cells. Escape of TRP-1–specific T cells from the thymus then enhances immune rejection of melanoma (Fig. 7B). These findings lend support to the proposition that immune-mediated control of tumor growth is modified by thymic expression of self-antigens that are also expressed in tumors (33, 34).

A recent study reported that Aire-deficient mice, if primed with irradiated melanoma cells, have increased immune response to melanoma antigens and have increased likelihood of tumor-free survival (19). We report here that Aire-deficient mice have an enhanced immune response to melanoma resulting in slower melanoma growth, even without priming with irradiated melanoma cells. Why the requirement for priming differs between the 2 studies is not clear, but may reflect differences in housing conditions or in the number of B16 cells inoculated per mouse (2 × 105 vs. 1 × 105). In both studies, an elevated antibody response to melanoma antigens was seen in Aire deficiency. Whether these antibodies participate in the immune rejection of melanoma, however, is unknown. Elevated autoantibodies against noncancer self-antigens have previously been described in Aire-deficient mice but do not seem to play an active role in autoimmune disease pathogenesis (35, 36).

Loss of Aire function has multiple effects on developing thymocytes. In addition to decreasing expression of a large number of tissue-specific self-antigens (37), Aire deficiency has also been proposed to downregulate the thymic chemokines CCR4 and CCR7 (38), to decrease thymic medullary accumulation of dendritic cells via decreased XCL1 expression (16), and to impede antigen processing and presentation (14). In this study, Aire’s role in upregulating TRP-1 expression in the thymus seems to be sufficient for mediating changes in TRP-1–specific CD4+ T-cell development in the thymus. Compared with Aire-deficient thymi, TRP-1 antigen-deficient (Tyrp1fl/fl) thymi exhibited similar defects in the negative selection of TRP-1–specific CD4+ T cells.

In this study, Aire deficiency significantly delayed melanoma growth in TRP-1 TCR Tg mice. This finding differs from a previous report that melanoma growth was only marginally delayed in TRP-1 TCR Tg mice with genetic mutation in TRP-1 (31). A potential explanation for this discrepancy is that TRP-1 is lacking not only in the thymus but also in the periphery (i.e., melanocytes) of TRP-1 TCR transgenic mice with a genetic mutation in TRP-1. Thus T cells are naïve to the TRP-1 antigen before introduction of B16 melanoma cells. In TCR transgenic mice deficient in Aire, on the other hand, TRP-1 is deficient in the thymus but not in the periphery. Thus, TRP-1 antigen-availability in melanocytes may prime TRP-1–specific T cells and allow for more effective rejection of melanoma cells.

In addition to promoting T-cell tolerance within the thymus, Aire also enforces self-tolerance within secondary lymphoid organs (30). We use thymic transplantation studies to show that immune tolerance toward B16 melanoma is conferred by Aire expression in the thymus and does not require extrathymic Aire expression. In contrast to our results, a previous study has reported that CD8+ T-cell tolerance toward tyrosinase, another shared vitiligo/melanoma antigen, is not mediated by tyrosinase expression in the thymus (21). Furthermore, extrathyMIC induction of T-cell tolerance toward tyrosinase has been shown to be Aire-independent (39). Thus, distinct tolerance mechanisms govern CD4+ T-cell responses to TRP-1 compared with CD8+ T-cell responses to tyrosinase.

To date, studies investigating the role of Aire in thymic negative selection have used mouse models involving forced expression of a ‘neo’-self-antigen [e.g., ovalbumin (OVA) and hen egg lysosome (HEL)] under tissue-specific promoters within the thymus (13, 14, 40, 41). Because these antigens are overexpressed foreign proteins, their expression pattern and levels may not mirror physiologic self-antigens (42). We show
Figure 6. Slower growth of B16 melanoma in Aire-deficient TRP-1 TCR Tg mice is associated with an increased density of TRP-1-specific tumor-infiltrating T cells. A, B16 melanoma cells were inoculated subcutaneously into AireGW/+ or Aire+/+ TRP-1 TCR Tg RAG1−/− mice. Representative tumors at day 26 after tumor inoculation are shown. B, tumor growth in either AireGW/+ or Aire+/+ TRP-1 TCR Tg RAG1−/− mice. n = 6 for both groups. Data shown are representative of 2 experiments carried out. C, survival curves of either AireGW/+ or Aire+/+ TRP-1 TCR Tg RAG1−/− mice. n = 6 for both groups. D, immunofluorescence
here that Aire deficiency results in reduced thymic expression of the endogenously expressed self-antigen, TRP-1. This loss of TRP-1 expression in the thymus directly results in the defective negative selection of TRP-1-specific CD4⁺ thymocytes. This finding is significant because it shows that Aire-mediated expression of endogenous thymic self-antigens is sufficient to drive efficient negative selection of self-reactive T cells.

The role of Aire deficiency in the generation of CD4⁺ Treg development in the thymus is controversial. Most studies have reported that Aire deficiency does not affect the absolute numbers of thymic CD4⁺ Tregs (13, 14, 43). Lei and colleagues, on the other hand, reported that thymic Tregs are decreased by 50% in Aire-deficient mice (16). Similar to the first group of studies, our study did not detect any difference in the absolute number of TRP-1-specific CD4⁺ Tregs developing in Aire-deficient thymi. A possible explanation for the differing conclusions is the methodologies used to quantify Tregs. Unlike the other studies, Lei and colleagues used immunofluorescence analysis of thymus sections to quantify Tregs within the medulla. It is also possible that Aire deficiency may alter Treg function in a qualitative manner. In our study, there seems to be an increased frequency of TRP-1 TCR Tg cells expressing low levels of Foxp3 in the periphery of Aire-deficient mice. The implications for this observation will require further investigation. However, it is noteworthy that CD4⁺ T cells expressing low levels of Foxp3 may play a role in autoimmunity (44). Notably, we show here that the ratio of effector:Tregs differ dramatically between Aire-deficient and wild-type TRP-1 TCR Tg mice, and an increased proportion of Tregs is inversely associated with antimalanoma immunity. This finding suggests an inhibitory role for TRP-1-specific Tregs in antimalanoma immunity.

Our finding that Aire-driven TRP-1 expression in the thymus results in clonal deletion of TRP-1-specific T cells has potential implications for designing immunotherapies in the treatment of metastatic melanoma. In metastatic melanoma, immunotherapeutic approaches result in complete responses in only a small subset of patients (4), and normal Aire-mediated self-tolerance mechanisms may contribute to the limited efficacy of these approaches (45). Pursuing strategies that modulate either Aire expression or TRP-1 expression in the thymus, for example, may allow increased escape of TRP-1-specific T-cell clones important for effective melanoma eradication. Alternatively, strategies that increase the number of TRP-1-reactive T cells in the periphery, such as adoptive transfer of TRP-1-reactive T cells, may help overcome Aire-mediated clonal deletion of these T cells in the thymus. Future studies to test these strategies will be of direct clinical relevance.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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