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Aire deficiency promotes TRP-1 specific immune rejection of melanoma

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Abstract

The thymic transcription factor 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. Further, transplantation of Aire-deficient thymic stroma was sufficient to confer more effective immune rejection of melanoma in an otherwise Aire wildtype 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.

Keywords

Aire; melanoma; TRP-1; negative selection; cancer antigens

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.

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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 two immune settings, the development of melanoma is regularly accompanied by vitiligo in a number of animal models (6). Additionally, 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 to 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 (mTECs) (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) (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). Additionally, 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 changes T cell responses to antigen-expressing tumors (20). Additionally, 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 demonstrate 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. Additionally, 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 anti-melanoma T cell response by decreased TRP-1 expression in the thymus.

Materials and Methods

Mice

Tyrp1$^{B–w}$ TRP-1 TCR Tg RAG$^{/}$mice (JAX stock number 008684) were purchased from The Jackson Laboratory. B6.Aire$^{GW/+}$ mice were backcrossed >10 generations from a mixed C57BL/6-129 line described in (22). To generate TRP-1-specific CD4^{+} TCR Tg mice in an Aire deficient background, male Tyrp1$^{B–w}$ TRP-1 TCR Tg RAG$^{–/–}$ mice were crossed with female B6.Aire$^{GW/+}$ mice. F1 male mice with genotype B6.Aire$^{GW/+}$ Tyrp1$^{B–w/+}$ TRP-1

TCR Tg $RAG^{+/-}$genotype were further crossed with $RAG^{-/-}$ mice. F2 mice with genotype $B6.Aire^{GW/+}$ $Tyrp1^{+/+}$ TRP-1 TCR Tg $RAG^{-/-}$ and $B6.Aire^{+/+}$ $Tyrp1^{+/+}$ TRP-1 TCR Tg $RAG^{-/-}$ mice 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 conducted with the approval of the Animal Use and Care Committees of the University of North Carolina, Chapel Hill.

Antibodies and Flow cytometry

Anti-CD4 (RM4-5), Anti-CD3 (145–2c11), anti-CD25 (PC61), anti-Vβ14 (14–2), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-Foxp3 (FJK-16s) were purchased from eBioscience. Anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG1.2), anti–MHC class II (I–A/I–E; M5/114.15.2) were purchased from BD Biosciences. Intracellular staining for cytokines was performed with the Cytofix/Cytoperm Intracellular Staining Kit (BD Biosciences) per manufacturer's instructions. Cells were stimulated with PMA/Ionomycin for 5 hours prior to antibody staining. FOXP3 Staining Buffer Set was used for FOXP3 intracellular staining according to manufacturer instructions (eBioscience). All samples were run on a Dako CyAn (Beckman-Coulter) flow cytometer.

B16 melanoma model

B16-F10 is a TRP-1^{+} C57BL/6-derived melanoma cell line that was kindly provided by Dr. Alan Fong (University of North Carolina, Chapel Hill). It was originally from ATCC, (Manassus, VA) and maintained in culture media as previously described (23, 24). Mice were injected subcutaneously with 1×10^{5} B16 melanoma cells in 100uL volume. Perpendicular tumor diameters were measured with calipers. Body weight and physiological status was monitored daily.

Realtime RT-PCR

Thymic stromal preparations and real-time RT-PCR were performed 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 performed as previously described (22). For bone marrow chimeras, bone marrow was harvested from the femurs of 6 weeks old $Tyrp1^{B–w}$ 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 (1×10^{7}) were injected retro-orbitally into each recipient mouse. Chimeras were aged for 10 weeks prior to analysis.

For thymic transplants, thymi from neonates were removed and cultured in Transwell plates for 7 days in 1.35 mM 2′-deoxyguanosine (2-dG) (Sigma-Aldrich) in complete DMEM to deplete hematopoietic cells. Thymi were washed in complete DMEM (without 2-dG) 2 hours prior to transplantation. Thymi were transplanted under the kidney capsule of B6.Nude recipients. Transplanted mice were aged for 10 weeks prior to 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-$1^{109–130}$ peptide (NCGTCRPG WRGAACNQKILTVR) was incubated overnight at 4C° in 96 well ELISA microwell plates (Xenopore) according to

the manufacturer's instructions. After blocking with 3% BSA in PBS, mouse sera were added at a 1:200 dilution in PBS and incubated for 2 h at room temperature. Goat anti-mouse 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. Respective positive controls (anti-TRP-1 antibody) and negative controls (no serum, antihuman IgG alone) were performed in parallel. Assays were performed at least twice with wells plated in 5 replicates for verification of results.

Immunofluorescence staining

Tumors from mice were harvested and fixed overnight in 10% formalin at 4C°, then stored in 70% ethanol. Tumors were embedded in paraffin for sectioning. Slides were stained overnight with anti-CD3 antibody, washed, and mounted with 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 $Tyrp1^{B-w}$ TRP-1 TCR Tg $RAG^{-/-}$ male mice were harvested and made into single-cell suspensions. Red blood cells were eliminated by ACK 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.

Statistics

PRISM 5.0 and Microsoft Office Excel software was used to analyze data (GraphPad Software, Inc.). Unpaired Student's t-tests were used to compare the differences between two groups. Survival curves were compared by Mann-Whitney U-test. P-values of ≤0.05 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 utilized a C57/BL6 mouse line harboring a dominant Aire G228W point mutation that results in hypomorphic Aire function ($B6.Aire^{GW/+}$ mice; (22)). Increased serum autoantibodies against melanoma cell extract were seen in 15–25 weeks old $B6.Aire^{GW/+}$ mice compared to wildtype ($B6.Aire^{+/+}$) littermate controls (Supp. Fig 1a, left). Consistent with these findings, increased serum autoantibodies against melanoma cell extract were also seen in 15–25 weeks old Aire knockout ($B6.Aire^{-/-}$) mice (Supp. Fig 1a,

right). These findings suggest that Aire deficiency promotes an autoimmune response against melanocyte antigens.

Since Aire-deficient mice demonstrate 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.Aire$^{GW/+}$ mice and followed tumor growth. B16 melanoma cells grew more slowly in B6.Aire$^{GW/+}$ mice compared with B6.Aire$^{+/+}$ littermate controls (Fig 1a and 1b; Supp. Fig 1b). Between Day 16 and Day 19, the average size of tumors was significantly reduced in B6.Aire$^{GW/+}$ compared to 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 to B6.Aire$^{+/+}$ mice (Fig 1b). Additionally, improved survival was seen in Aire-deficient B6.Aire$^{GW/+}$ and B6.Aire$^{-/-}$ mice inoculated with B16 melanoma cells compared to B6.Aire$^{+/+}$ littermate controls (Fig 1c).

The decreased B16 melanoma growth in Aire-deficient mice was associated with increased T cell immune infiltration within the tumors. We utilized 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.Aire$^{GW/+}$ mice compared with B6.Aire$^{+/+}$ mice (Fig 1d and 1e). 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.Aire$^{GW/+}$ mice. We pooled together lymphocytes from the tumors of 6 B6.Aire$^{GW/+}$ and 6 wildtype littermate controls to collect a sufficient number of events to analyze. An increased frequency CD4^{+} and CD8^{+} T lymphocytes with an activated/memory phenotype (CD62L^{low} CD44^{high}) were found within melanoma of B6.Aire$^{GW/+}$ mice compared to wildtype littermates (Fig 2a). An increased frequency of CD62L^{low} CD44^{high} CD4^{+} and CD8^{+} T cells were also seen in the spleen and tumor-draining lymph node (Supp. Fig 2).

We next assessed the ability of tumor-infiltrating lymphocytes to secrete pro-inflammatory cytokines by intracellular cytokine staining. We evaluate the ability of T cells to secrete IL-2 and IFNγ, since both cytokines have been implicated in rejection of tumors by T cells (27–29). An increased frequency of IL-2 and IFNγ secreting CD4^{+} and CD8^{+} tumor-infiltrating lymphocytes were detected in B6.Aire$^{GW/+}$ mice compared to wildtype littermate controls (Fig 2b and 2c). An increased frequency of CD4^{+} and CD8^{+} T lymphocytes secreting IL-2 and IFNγ was also seen in spleen and tumor-draining lymph nodes of B6.Aire$^{GW/+}$ mice compared to wildtype littermates (Fig 2d and 2e). Importantly, we did not observe a difference in the frequency of memory/activated or cytokine-secreting T cells in B6.Aire$^{GW/+}$ mice prior to tumor inoculation (Supp. Fig 3a-3d). The increased immune activation in B6.Aire$^{GW/+}$ mice, therefore, is specific to the anti-tumor response and does not represent non-specific 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.Aire$^{GW/+}$ mice or wildtype littermates into athymic nude (Foxn1$^{-/-}$; B6.Nude) mice. The reconstituted mice have wildtype Aire expression in the periphery, but are either Aire-deficient or Aire-wildtype 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 post transplantation. No significant difference in CD4$^+$ and CD8$^+$ T cell reconstitution was found between recipients of B6.Aire$^{GW/+}$ or B6.Aire$^{+/+}$ thymi (Supp. Fig 4).

After confirming immune reconstitution in transplanted mice, we inoculated B16 melanoma cells and monitored tumor growth (Fig 3a). Between Days 9 and 19 post inoculation, melanoma growth was significantly decreased in mice reconstituted with Aire-deficient thymi compared to wildtype thymi (Fig 3b). This decreased growth was associated with increased numbers of CD3$^+$ tumor infiltrating lymphocytes per area of tumor (mm^2) (Fig 3c and 3d). Additionally, an increased frequency of IL-2 and IFNγ-secreting CD4$^+$ and CD8$^+$ tumor infiltrating lymphocytes were seen in mice reconstituted with Aire-deficient thymi (Fig 3e and 3f). An increased frequency of IL-2 and IFNγ-secreting CD4$^+$ and CD8$^+$ splenocytes was also observed in mice reconstituted with Aire-deficient thymi (Fig 3g and 3h). An enhanced anti- tumor 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.Aire$^{GW/+}$ mice and B6.Aire$^{+/+}$ mice were isolated and relative expression levels of three 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 of TRP-1 and tyrosinase was significantly decreased in B6.Aire$^{GW/+}$ 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.Aire$^{GW/+}$ mice have increased serum autoantibodies against TRP-1, suggesting a break in self-tolerance toward this antigen in Aire-deficient mice (Fig 4b).

We utilized an MHC class II-restricted TRP-1 specific T cell receptor (TCR) transgenic mouse model (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 ($Tyrp1^{B-w}$), approximately 15% of lymphocyte-gated cells were single positive (SP) for CD4 (Fig 4c, left panel). In thymi in which cognate antigen is expressed ($Tyrp1^{+/+}$), on the other hand, the frequency of CD4 SP cells is significantly decreased (1–2%; Fig 4c, middle panel), indicating efficient negative selection of this population. This change was also seen with the frequency of clonotype specific Vβ14+ CD4SP cells among total thymocytes (Fig. 4d). In addition to decreased frequency of CD4 SP cells, the absolute number of CD4 SP thymocytes was also significantly decreased [9×10^5 cells in $Tyrp1^{B-w}$ thymi compared to 3×10^4 cells in $Tyrp1^{+/+}$ thymi (Fig 4e)]. Thus, consistent with a previous report (31), effective negative selection of TRP-1 TCR Tg $RAG^{-/-}$ cells occurs in thymi expressing normal levels of TRP-1 antigen.

Since 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 $RAG^{-/-}$ cells. We therefore generated TRP-1 TCR Tg $RAG^{-/-}$ mice on an Aire deficient background ($B6.Aire^{GW/+}$ TRP-1 TCR Tg $RAG^{-/-}$ mice) and characterized T cell development within the thymi of these mice. The frequency of CD4 SP cells was significantly greater in Aire-deficient thymi compared to thymi expressing normal levels of TRP-1 antigen (Fig 4c, compare middle and right panels). Similar trends were also seen when the frequency of clonotype specific Vα14+ CD4SP T cells were assessed as a percent of total thymocytes (Fig 4d). The absolute number of CD4 SP cells was also significantly greater in Aire-deficient thymi (1×10^6) compared to thymi expressing normal levels of TRP-1 antigen (3×10^4 cells; Fig 4e). Splenic $CD4^+$ T cells reflected the findings in the thymus (Fig 4f-h).

To investigate thymic negative selection using an alternative approach, we performed bone marrow chimera experiments in which $Tyrp1^{B-w}$ TRP-1 TCR Tg $RAG^{-/-}$ bone marrow was transplanted into lethally-irradiated $B6.Aire^{GW/+}$ or $B6.Aire^{+/+}$ recipients (Supp. Fig 5). Within these chimeric mice, TRP-1 specific T cells would develop in either an Aire-deficient or Aire wildtype thymic stromal environment. Increased frequency of CD4 SP cells was seen in Aire deficient recipient thymi (approximately 25%) compared with wildtype recipient thymi (approximately 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 $CD4^+$ $FOXP3^+$ regulatory T cells (Tregs) are generated 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 $B6.Aire^{GW/+}$ and $B6.Aire^{+/+}$ mice. Although the percentage of FOXP3+ Tregs among $CD4^+$ SP thymocytes is decreased in $B6.Aire^{GW/+}$ TRP-1 TCR Tg $RAG^{-/-}$ thymi compared to $B6.Aire^{+/+}$ TRP-1 TCR Tg $RAG^{-/-}$ mice (Fig 5a and 5b), 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 wildtype TRP-1 TCR Tg $Rag^{-/-}$ thymi (Fig 5a and 5d). Additionally, the frequency of FOXP3+ Tregs is also decreased in the spleen of Aire deficient mice, but the absolute numbers are increased (Supp. Fig 6a, 6b and 6c).

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 (Supp. Fig 6a and d). Since 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:regulatory T cell ratio was higher in both thymus and spleen of Aire deficient mice (Fig 5d). This increased ratio suggests that the balance of effector:regulatory T cells are tipped toward increased effectors in Aire-deficient mice.

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

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 $B6.Aire^{GW/+}$ TRP-1 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-wildtype controls between days 19–26 (Fig 6a and 6b). Additionally, Aire-deficient mice inoculated with B16 melanoma showed significantly increased survival compared to Aire-wildtype controls (Fig 6c).

Decreased B16 melanoma growth was associated with increased density of tumor infiltrating T cells in $B6.Aire^{GW/+}$ 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 to Aire wildtype controls (Fig 6d and 6e). This increased density of tumor infiltrating lymphocytes was associated with increased absolute numbers of activated/memory ($CD62L^{low}$ $CD44^{high}$) TRP-1 specific $CD4^+$ T cells in the spleen and tumor-draining lymph nodes (Fig 6f). Additionally, 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 pro-inflammatory cytokines.

As shown in Fig 5e, the ratio of Effector:Regulatory T cells 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 performed adoptive transfers of T cells into RAG deficient recipients at the two effector to regulatory T cell ratios seen in wildtype (Effector:Regulatory 1:3) and Aire deficient (Effector:Regulatory 2:1) mice. Adoptive transfer of T cells at the effector to regulatory T cell ratio in spleen of Aire deficient mice protected against melanoma outgrowth, whereas adoptive transfer of T cells at the effector to regulatory T cell ratio in spleen of wildtype mice did not (Fig 6h). These findings suggest that the increased proportion of Tregs may prevent efficient anti-melanoma immunity in Aire wildtype mice.

Discussion

This study defines a novel pathway by which Aire deficiency enhances T cell-mediated immune rejection of melanoma. In wildtype 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 two studies is not clear, but may reflect differences in housing conditions or in the number of B16 cells inoculated per mouse (2×10^5 versus 1×10^5). 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 non-cancer self-antigens have previously been described in Aire-deficient mice, but do not appear 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 appears to be sufficient for mediating changes in TRP-1 specific $CD4^+$ T cell development in the thymus. Compared to Aire-deficient thymi, TRP-1 antigen deficient ($Tyrp1^{B-w}$) 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 prior to 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 utilize 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 to $CD8^+$ T cell responses to tyrosinase.

To date, studies investigating the role of Aire in thymic negative selection have utilized mouse models involving forced expression of a "neo"-self-antigen [e.g. ovalbumin (OVA) and hen egg lysomzyme (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 demonstrate 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 demonstrates 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 et al, on the other hand, reported that thymic Tregs are decreased by 50% in Airedeficientmice (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 et al utilized 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 appears 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:Regulatory T cells differ dramatically between Aire deficient and wildtype TRP-1 TCR Tg mice, and an increased proportion of Tregs is inversely associated with anti-melanoma immunity. This finding suggests an inhibitory role for TRP-1 specific Tregs in anti-melanoma 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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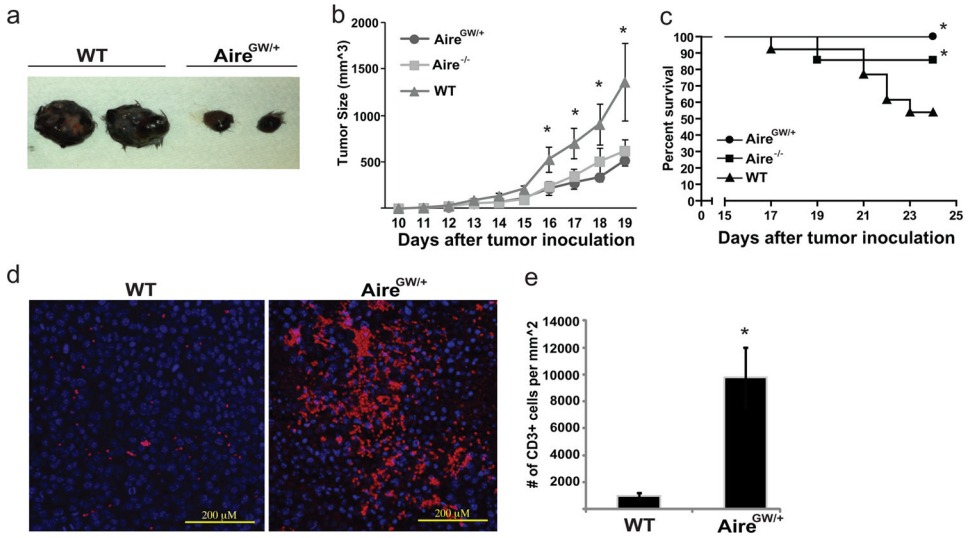

Figure 1. Slower growth of B16 melanoma in Aire-deficient mice is associated with increased density of tumor-infiltrating T cells

(a) B16 melanoma cells were inoculated subcutaneously on the flanks of B6.Aire$^{GW/+}$ (Aire$^{GW/+}$) mice or B6.Aire$^{+/+}$ (WT) mice. Representative tumors from Aire$^{GW/+}$ mice or Aire$^{+/+}$ mice at day 18 post inoculation are shown. (b) Tumor growth in either B6.Aire$^{GW/+}$, B6.Aire$^{-/-}$ (Aire$^{-/-}$) or B6.Aire$^{+/+}$ mice. n=7 for all groups. Data shown is representative of 3 independent experiments performed. (c) Survival curves of either Aire$^{GW/+}$, Aire$^{-/-}$, or Aire$^{+/+}$ mice. n=6 for all groups. (d) Representative immunofluorescence staining for CD3 (Red) and DAPI for melanoma cell nuclei (Blue) on tumor sections from Aire$^{GW/+}$ mice or Aire$^{+/+}$ mice at day 18 after B16 melanoma inoculation. (e) Average densities of CD3^{+} cells per tumor area (mm^2) in tumors inoculated into either Aire$^{GW/+}$ or Aire$^{+/+}$ mice. 4 sections were evaluated for each group and 6 areas from each section were randomly selected. *P<0.05, error bars represent SEM.

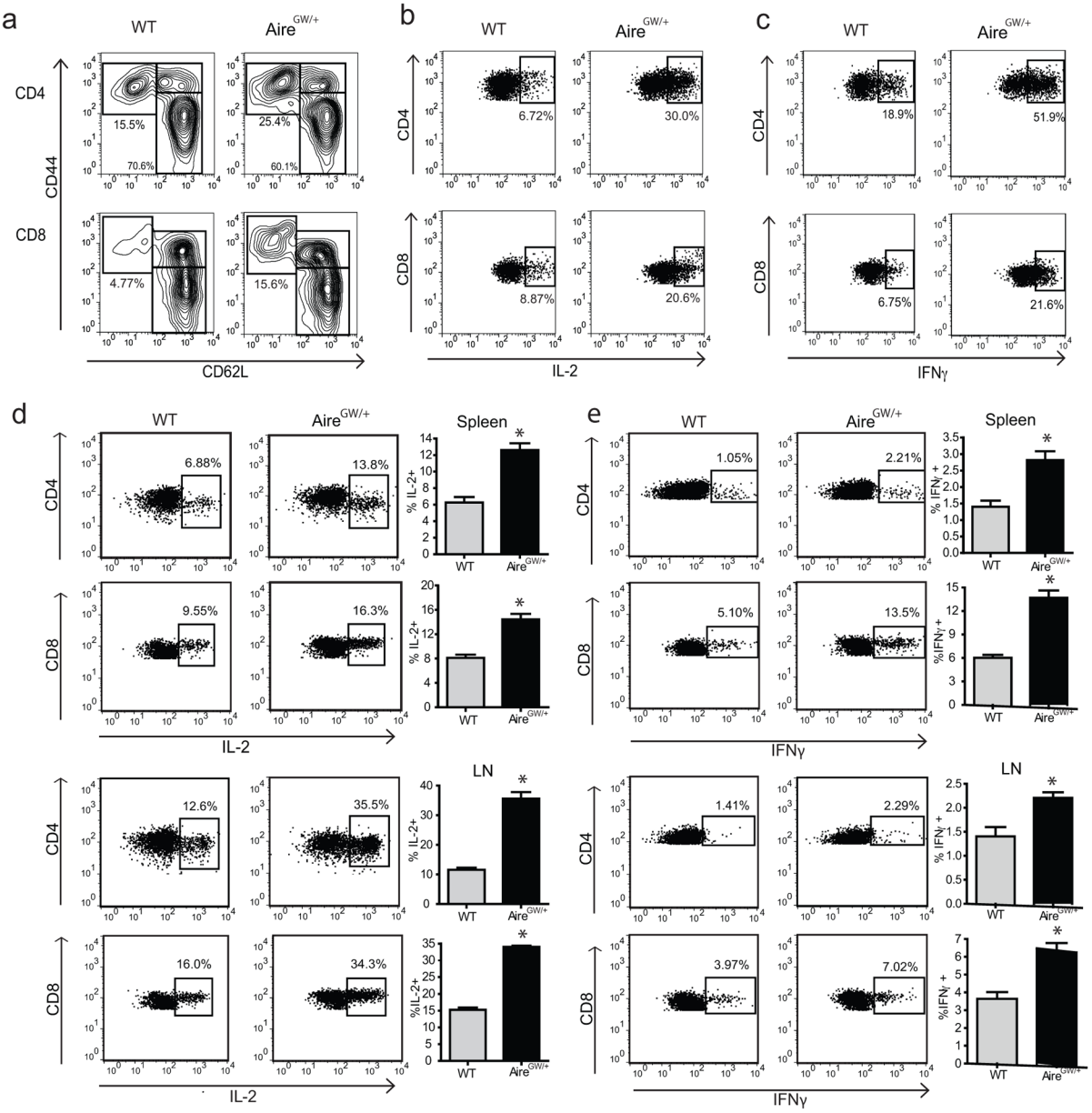

Figure 2. Aire-deficient mice inoculated with B16 melanoma have activated T cells with increased pro-inflammatory cytokine production

(a) Flow cytometry plots of CD62L and CD44 expression in tumor infiltrating lymphocytes from Aire$^{GW/+}$ or 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 panels. n=4 for both groups. Data shown is representative experiment of 3 experiments. *P<0.05, error bars represent SEM.

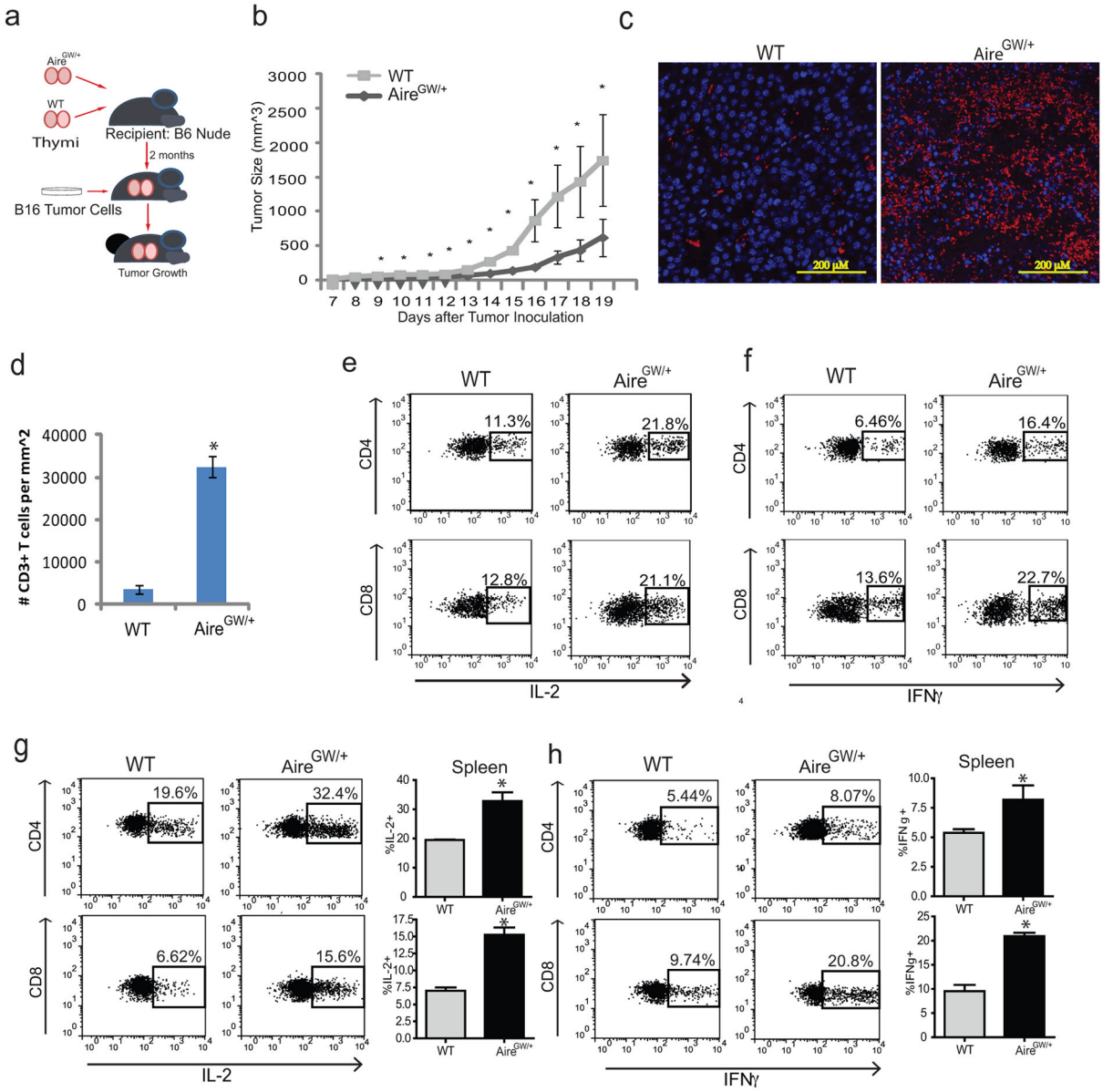

Figure 3. Augmented response against melanoma maps to Aire difference in thymic stroma

(a) Scheme for thymic transplantation experiment. Thymic stroma from Aire$^{GW/+}$ mice or 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 WT 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 WT thymi. 4 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 WT thymic stroma. (e and f) Tumor infiltrating lymphocytes were isolated from mice reconstituted with Aire$^{GW/+}$ or WT thymi, 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 panels. n=4 for both groups. *P<0.05, error bars represent SEM.

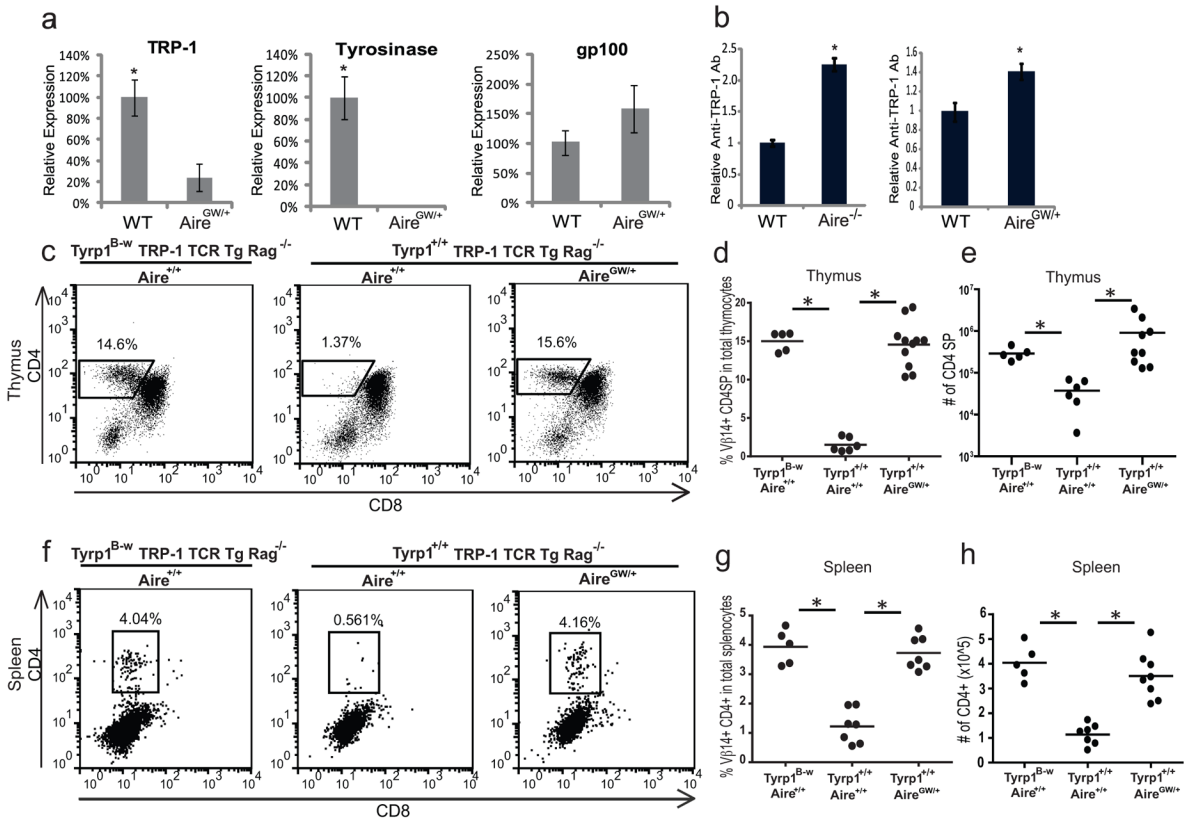

Figure 4. Aire promotes TRP-1 expression and negative selection of TRP-1 specific T cells in the thymus

(a) Relative mRNA expression of TRP-1, tyrosinase and silver/gp100 evaluated by real-time RT-PCR in sorted mTECs from $Aire^{GW/+}$ or $Aire^{+/+}$ thymi. Data are representative of 4 independent experiments. (b) Relative amount of TRP-1 autoantibodies in sera from $Aire^{GW/+}$, $Aire^{-/-}$ and $Aire^{+/+}$ mice determined by ELISA. n=5 in each group. (c) Representative plots of lymphocytes expressing CD4 and CD8 in thymus of $Tyrp1^{B-w}$ TRP-1 TCR Tg $RAG^{-/-}$, $Aire^{GW/+}$ TRP-1 TCR Tg $RAG^{-/-}$, or $Aire^{+/+}$ TRP-1 TCR Tg $RAG^{-/-}$ mice. (d and e) Cumulative data for percentage of $V\beta14^+$ CD4SP T cells in total thymocytes (d) and absolute number of CD4 SP thymocytes (e). Each shape represents an individual mouse. (f) Representative plots of CD4 and CD8 expression in spleen of $Tyrp1^{B-w}$ TRP-1 TCR Tg $RAG^{-/-}$, $Aire^{GW/+}$ TRP-1 TCR Tg $RAG^{-/-}$, or $Aire^{+/+}$ TRP-1 TCR Tg $RAG^{-/-}$ mice. (g and h) Cumulative data for percentage of $V\beta14^+$ $CD4^+$ T cells in total splenocytes (g) and absolute number of $CD4^+$ splenocytes (h). Error bars represent SEM.

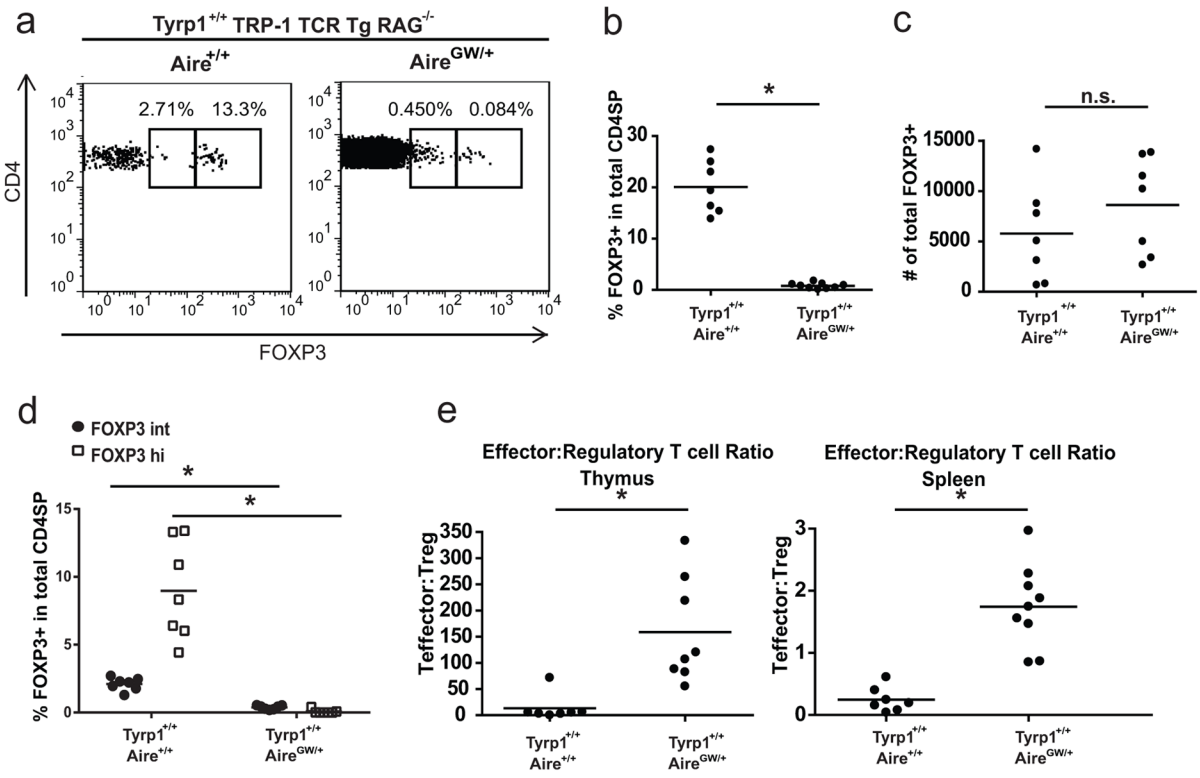

Figure 5. Aire does not affect numbers of TRP-1 specific CD4$^+$ FOXP3$^+$ Tregs in the thymus

(a) Representative flow cytometry plots of CD4 and FOXP3 expression in CD4$^+$ thymocytes from Aire$^{GW/+}$ 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 CD4SP 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 CD4SP thymocytes (e) Cumulative data of effector (CD4$^+$ FOXP3$^-$) versus regulatory T cell (CD4$^+$ FOXP3$^+$) ratios in thymocytes (left panel) and splenocytes (right panel) from Aire$^{+/+}$ or Aire$^{GW/+}$ TRP-1 TCR Tg RAG$^{-/-}$ mice.

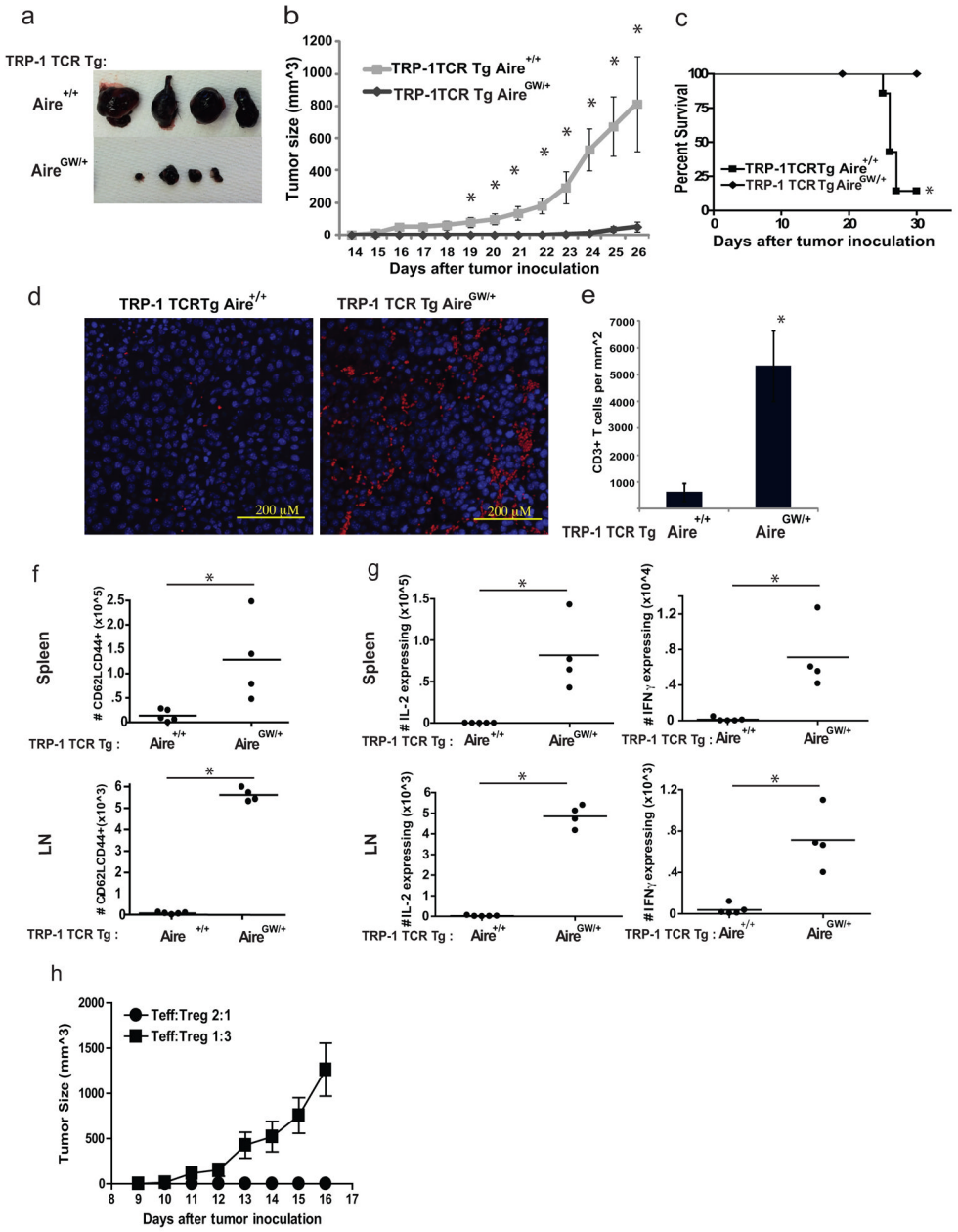

Figure 6. Slower growth of B16 melanoma in Aire-deficient TRP-1 TCR Tg mice is associated with an increased density of TRP-1 specific tumor-infiltrating T cells

(a) B16 melanoma cells were inoculated subcutaneously into Aire$^{GW/+}$ or Aire$^{+/+}$ TRP-1 TCR Tg RAG$^{-/-}$mice. Representative tumors at day 26 after tumor inoculation are shown. (b) Tumor growth in either Aire$^{GW/+}$ or Aire$^{+/+}$ TRP-1 TCR Tg RAG$^{-/-}$ mice. n=6 for both groups. Data shown is representative of 2 experiments performed. (c) Survival curves of either Aire$^{GW/+}$ or Aire$^{+/+}$ TRP- 1 TCR Tg RAG$^{-/-}$ mice. n=6 for both groups. (d) Immunofluorescence staining for CD3 (Red) and DAPI staining for tumor nuclei (Blue) on B16 melanoma sections from Aire$^{GW/+}$ or Aire$^{+/+}$ TRP-1 TCR Tg RAG$^{-/-}$ mice at day 26 after tumor inoculation. 4 tumor sections were evaluated for each group and 6 areas from each section were randomly selected. (e) Average densities of CD3^{+} cells per tumor area (mm^2) in either Aire$^{GW/+}$ or Aire$^{+/+}$ TRP-1 TCR Tg RAG$^{-/-}$ mice. (f) Absolute numbers of

activated ($CD62L^{low}$ $CD44^{high}$) $CD4^+$ T cells from spleen and draining lymph nodes (LN) as evaluated by flow cytometry. Each shape represents individual mouse. (g) Absolute numbers of $CD4^+$ T cells expressing IL-2 and IFN-γ from spleen and draining lymph nodes (LN) as evaluated by flow cytometry. Each shape represents individual mouse. (h) Melanoma growth in RAG deficient mice receiving 2×10^5 $CD4^+CD25^-$ effector T cells and variable $CD4^+CD25^+$ regulatory T cells at the indicated ratios of effector to regulatory T cells. *P<0.05, error bars represent SEM. *P<0.05, error bars represent SEM.

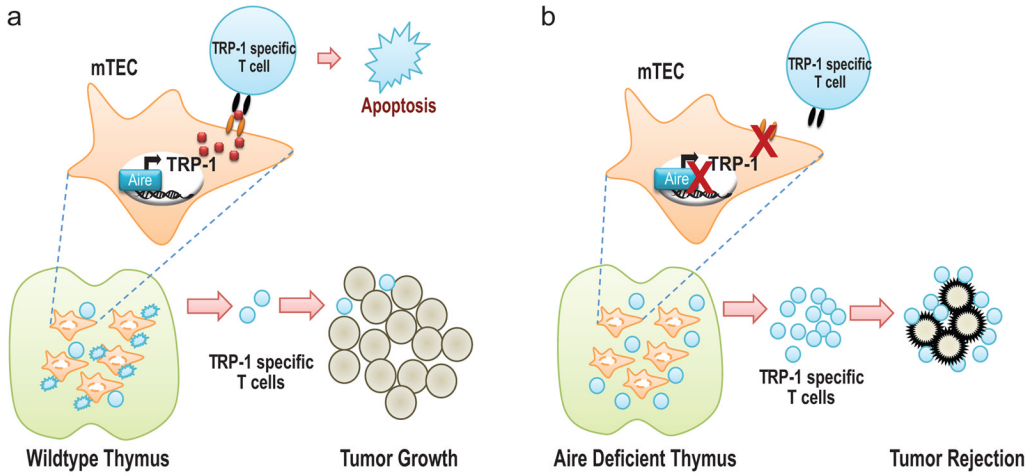

Figure 7. Model of how Aire deficiency elicits a more robust immune response against melanoma

(a) Aire normally induces TRP-1 expression in thymic mTECs. Presentation of TRP-1 to developing T cells results in deletion of TRP-1 specific T cells via apoptosis. (b) Aire deficiency results in decreased TRP-1 expression in mTECs. Lack of TRP-1 presentation to TRP-1 specific T cells allows escape of these T cells from negative selection and enhances the immune response against melanoma.