Enhancement of an anti-tumor immune response by transient blockade of central T cell tolerance

Imran S. Khan,1 Maria L. Mouchess,1 Meng-Lei Zhu,3,4 Bridget Conley,3,4 Kayla J. Fasano,1 Yafei Hou,2 Lawrence Fong,2 Maureen A. Su,3,4 and Mark S. Anderson1

Thymic central tolerance is a critical process that prevents autoimmunity but also presents a challenge to the generation of anti-tumor immune responses. Medullary thymic epithelial cells (mTECs) eliminate self-reactive T cells by displaying a diverse repertoire of tissue-specific antigens (TSAs) that are also shared by tumors. Therefore, while protecting against autoimmunity, mTECs simultaneously limit the generation of tumor-specific effector T cells by expressing tumor self-antigens. This ectopic expression of TSAs largely depends on autoimmune regulator (Aire), which is expressed in mature mTECs. Thus, therapies to deplete Aire-expressing mTECs represent an attractive strategy to increase the pool of tumor-specific effector T cells. Recent work has implicated the TNF family members RANK and RANK-Ligand (RANKL) in the development of Aire-expressing mTECs. We show that in vivo RANKL blockade selectively and transiently depletes Aire and TSA expression in the thymus to create a window of defective negative selection. Furthermore, we demonstrate that RANKL blockade can rescue melanoma-specific T cells from thymic deletion and that persistence of these tumor-specific effector T cells promoted increased host survival in response to tumor challenge. These results indicate that modulating central tolerance through RANKL can alter thymic output and potentially provide therapeutic benefit by enhancing anti-tumor immunity.

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Abbreviations used: Aire, autoimmune regulator; cTEC, cortical thymic epithelial cell; IRBP, interphotoreceptor retinoid-binding protein; mTEC, medullary thymic epithelial cell; OPG, Osteoprotegerin; TSA, tissue-specific antigen.

1Diabetes Center and 2Division of Hematology/Oncology, Department of Medicine, University of California, San Francisco, San Francisco, CA 94143
3Department of Pediatrics and 4Department of Microbiology/Immunology, School of Medicine, and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599

Medullary thymic epithelial cells (mTECs) contribute to self-tolerance through the ectopic expression of tissue-specific antigens (TSAs) in the thymus (Derbinski et al., 2001; Anderson et al., 2002; Metzger and Anderson, 2011). This TSA expression in mTECs is largely dependent on autoimmune regulator (Aire), which is expressed in mature mTECs (Gäbler et al., 2007; Gray et al., 2007; Metzger and Anderson, 2011). Through the recognition of TSAs, developing autoreactive T cells are either negatively selected from the pool of developing thymocytes or recruited into the regulatory T (T reg) cell lineage (Liston et al., 2003; Anderson et al., 2005; DeVoss et al., 2006; Shum et al., 2009; Taniguchi et al., 2012; Malchow et al., 2013). The overall importance of this process is underscored by the development of a multi-organ autoimmune syndrome in patients or mice with defective AIRE expression (Consortium, 1997; Nagamine et al., 1997; Anderson et al., 2002).

Although central tolerance provides protection against autoimmunity, this process also represents a challenge for anti-tumor immunity (Kyewski and Klein, 2006; Malchow et al., 2013). Because many of the TSAs expressed in the thymus are also expressed in tumors, high-affinity effector T cells capable of recognizing tumor self-antigens may normally be deleted in the thymus (Bos et al., 2005; Cloosen et al., 2007; Träger et al., 2012; Zhu et al., 2013). Transiently suppressing central tolerance by depleting mTECs or modulating Aire expression may provide a...
therapeutic window for the generation of T cells capable of recognizing tumor self-antigens. Many current cancer immune therapies rely on activating relatively weak tumor-specific T cell responses through modulating peripheral tolerance (Swann and Smyth, 2007; Chen and Mellman, 2013). In contrast, manipulation of central tolerance has the potential to increase the pool and affinity of effector T cells that can recognize and contribute to effective anti-tumor responses. Furthermore, such high-affinity, self-reactive T cells may be more resistant to peripheral tolerance mechanisms that typically restrain an anti-tumor response (Swann and Smyth, 2007). Thus, the development of methods that selectively and transiently deplete Aire-expressing mTECs may be an attractive method to enhance tumor-specific immune responses.

Previous work has identified agents that can inhibit the growth and development of TECs such as corticosteroids, cyclosporine, and some inflammatory cytokines (Anz et al., 2009; Fletcher et al., 2009). Despite their clear inhibitory effects on TECs, however, these agents do not appear to have selectivity for blocking mTEC development. Interestingly, recent studies have demonstrated a role for TNF family member pairs RANK–RANKL and CD40–CD40L in the embryological development of Aire+ mTECs (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka et al., 2008; Roberts et al., 2012). Recent work has also demonstrated that mTECs in particular have a relatively fast turnover in adult mice with an estimated half-life of ~2 wk (Gäbler et al., 2007; Gray et al., 2007). Given these findings, we speculated that in vivo blockade of RANKL in adult hosts could both selectively and transiently inhibit the development and turnover of mTECs with potential to alter central T cell tolerance. To this end, we performed in vivo RANKL blockade in adult mice and investigated its effects on both TECs and developing thymocytes. We show that anti-RANKL treatment not only depleted mTECs but could also be used therapeutically to break central tolerance and, as a result, increase the generation of tumor-specific T cells.

RESULTS AND DISCUSSION
Depletion of mTECs with RANKL blockade
The RANK–RANKL signaling pathway is important for mTEC development, but it remains unclear what impact perturbation of this pathway might have on the adult thymus. Previous work has linked the RANK–RANKL pathway to the development of Aire-expressing mTECs (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka et al., 2008; Roberts et al., 2012), and we hypothesized that treating mice with blocking anti-RANKL antibody would decrease Aire+ mTECs.
We treated wild-type mice with either anti–RANKL or isotype control antibody for 2 wk and harvested their thymi for analysis. Interestingly, although mice treated with anti–RANKL showed only a modest loss of cortical thymic epithelial cell (cTEC) cellularity, they exhibited a severe depletion of >80% of mTECs (Fig. 1 A). Using MHC II and Aire as markers of mTEC maturation (Gäbler et al., 2007; Gray et al., 2007), we further analyzed the immature mTEC<sup>lo</sup> (MHC II<sup>lo</sup> Aire<sup>+</sup>), intermediate mTEC<sup>hi</sup> (MHC II<sup>hi</sup> Aire<sup>-</sup>), and mature Aire<sup>-</sup> mTEC subsets (MHC II<sup>hi</sup> Aire<sup>-</sup>). The relative mTEC composition in anti–RANKL–treated mice revealed a substantial loss of Aire<sup>-</sup> and mTEC<sup>hi</sup> cells along with enrichment of the remaining mTEC<sup>lo</sup> cells (Fig. 1 B). Although absolute numbers of mTECs were decreased across all mTEC subsets, mTEC depletion was mostly due to the loss of >90% of all Aire<sup>-</sup> and mTEC<sup>hi</sup> cells (Fig. 1 B). Immunostaining revealed a slight decrease in the density of keratin-5<sup>+</sup> (K5) cells in the medulla of anti–RANKL–treated mice, whereas Aire<sup>-</sup> cells were nearly undetectable (Fig. 1 C). Importantly, staining for keratin-8 (K8) and K5 showed that the overall corticomedullary thymic architecture was preserved despite the loss of mature mTECs (Fig. 1 C). Furthermore, consistent with the loss of the Aire<sup>-</sup> and mTEC<sup>hi</sup> subsets, Aire–dependent TSA gene expression in sorted mTECs was decreased in anti–RANKL–treated mice (Fig. 1 D).

Next, we characterized the impact of anti–RANKL–mediated mTEC depletion on thymocyte selection. In the polyclonal T cell repertoire of wild-type mice treated with anti–RANKL, we observed a modest increase in frequencies of both CD4 single-positive (SP) and CD8 SP thymocytes, consistent with a lack of negative selection (Fig. 1 E). Importantly, anti–RANKL–treated mice showed only a slight reduction in the frequency of double-positive thymocytes while absolute numbers were maintained, confirming normal positive selection. In addition, total thymocyte numbers were also maintained in mice treated with anti–RANKL (Fig. 1 E). Furthermore, anti–RANKL–treated mice showed a 50% reduction of Foxp3<sup>+</sup> T reg cells within the CD4 SP subset (Fig. 1 F).

Given the dramatic impact of RANKL blockade on mTECs, we sought to determine whether this effect could be reversed in the context of increased RANK signaling. Osteoprotegerin (OPG; Tnfrsf11b) is a soluble decoy receptor for RANKL and its role as a negative regulator of RANK signaling has been well described in bone physiology (Kearns et al., 2007). Notably, thymi from anti–RANKL– and corticomedullary thymic architecture despite these changes in TEC cellularity and mTEC composition (Fig. 2 D).

Collectively, these data suggest that RANK–RANKL–OPG interactions regulate both TEC cellularity and mTEC maturation, and are particularly important for the induction of Aire<sup>-</sup> mTECs. Furthermore, anti–RANKL treatment selectively targeted mature mTECs and altered negative selection without affecting the development of new thymocytes.

**Regeneration of mTECs after withdrawal of anti–RANKL**

We next examined the kinetics of mTEC recovery after withdrawal from anti–RANKL blockade. After a 2-wk treatment window, we harvested mice at 2-wk intervals and observed gradual recovery of the mTEC<sup>hi</sup> and Aire<sup>-</sup> subsets (Fig. 3, A and B). By 10 wk, we observed the complete recovery of all mTEC subsets and a normal level of Aire expression after anti–RANKL treatment (Fig. 3 B). Interestingly, the pattern of mTEC recovery appeared consistent with published reports of mTEC<sup>lo</sup> cells giving rise to mTEC<sup>hi</sup> cells before the induction of Aire<sup>-</sup> mTECs (Fig. 3 B; Gäbler et al., 2007; Gray et al., 2007; Rossi et al., 2007). Notably, thymi from anti–RANKL– and

"Fig. 2. Increased Aire<sup>-</sup> mTECs in OPG<sup>-/-</sup> mice." (A) Quantitative PCR analysis of Aire and OPG gene expression in MHC II<sup>lo</sup> and MHC II<sup>hi</sup> mTECs sorted from wild-type B6 mice. Results standardized to Cyclophilin A and normalized to MHC II<sup>lo</sup>, with error bars depicting mean ± SD. (B) Absolute numbers of mTECs and cTECs in OPG<sup>-/-</sup> (black) and OPG<sup>-/-</sup> (red) mice were enumerated by flow cytometry. Bar graphs of total cell numbers are depicted by mean ± SEM. (C) Representative flow cytometry plots of mTECs in B showing relative composition of indicated mTEC subsets. Values depict mean ± SEM. Bar graphs (right) depict total cell numbers of each mTEC subset and indicate mean ± SEM. (D) Immunostaining for keratin-8 (green) and keratin-5 (red) on frozen thymic sections from OPG<sup>-/-</sup> and OPG<sup>-/-</sup> mice. Data are representative of at least three independent experiments with at least three mice per group. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001, Student’s t test. Bars, 500 µm."
isotype-treated mice at 10 wk were indistinguishable by immunostaining (Fig. 3C). Thus, anti-RANKL–mediated mTEC depletion is a transient phenomenon with return of normal thymic composition after withdrawal of antibody treatment.

Manipulation of thymic negative selection with RANKL blockade

To further characterize the impact of anti-RANKL treatment on negative selection, we first used the OT-II CD4+ TCR x RIP-mOVA double transgenic mouse model. RIP-mOVA transgenic mice express a membrane form of OVA under the control of the rat insulin promoter which results in its expression in both the pancreatic islets and in mTECs (Anderson et al., 2005). When the OT-II CD4+ TCR transgenic line is crossed to the RIP-mOVA transgenic line, OVA–specific OT-II-TCR+ cells are deleted in the thymus (Anderson et al., 2005). We treated both OT-II and OT-II x RIP-mOVA mice with either anti-RANKL or isotype control antibody and performed thymocyte analysis. Consistent with previous reports, OT-II x RIP-mOVA mice treated with control antibody showed a significant...
reduction in the proportions of CD4 SP thymocytes and also had decreased frequencies and numbers of OT-II T cells (Fig. 4, A and B; Anderson et al., 2005). In contrast, thymocyte profiles of anti-RANKL–treated OT-II x RIP-mOVA mice were indistinguishable from that of OT-II mice, demonstrating that RANKL blockade prevented thymic deletion of OT-IHT cells yet allowed their positive selection (Fig. 4, A and B). We also observed a loss of thymic T reg cell development in these mice, which suggested a loss of cognate antigen (OVA) expression from mTECs within the thymus (Fig. 4 C).

To expand these observations to the polyclonal T cell repertoire, we analyzed the development of Aire–dependent autoreactive T cells using a tetramer enrichment protocol. Previously, we had shown that T cells specific for the self-antigen interphotoreceptor retinoid-binding protein (IRBP) are deleted in thymus of Aire+/− mice, whereas these cells escape deletion in Aire−/− mice and cause autoimmune uveitis (DeVoss et al., 2006; Taniguchi et al., 2012). Through the use of an IRBP peptide class II tetramer, P2-I-Aβ, such autoreactive CD4+ T cells can be detected in Aire−/− mice. Given both the severe depletion of Aire+ mTECs and the loss of thymic IRBP expression in anti-RANKL–treated mice, we hypothesized that P2-I-Aβ–specific T cells could be detected in treated mice. After treating wild-type mice with anti-RANKL antibody, we immunized mice with a MHC II–binding IRBP peptide epitope (P2) to expand T cells for detection. 10 d after immunization, lymph nodes and spleen were pooled for the enumeration of CD4+ P2-I-Aβ–specific T cells by flow cytometry. Consistent with the loss of Aire+ mTECs, tetramer analysis of anti-RANKL–treated mice revealed an expansion of CD4+ P2-I-Aβ–specific T cells.
(Fig. 4 D). Overall, these data show a clear defect in negative selection associated with the loss of Aire+ mTECs in anti-RANKL–treated mice.

Treatment with anti-RANKL enhances anti-tumor immunity

Given its ability to selectively block mTECs and central tolerance, we next sought to determine whether anti-RANKL treatment could be used to therapeutically enhance anti-tumor immunity. Previous work has shown that many tumor self-antigens are expressed by mTECs as TSAs, and that high-affinity T cells capable of recognizing these tumor self-antigens are efficiently deleted in the thymus (Bos et al., 2005; Träger et al., 2012; Zhu et al., 2013). To test whether anti-RANKL treatment could rescue tumor self-antigen–specific T cells from thymic deletion, we used the TRP-1 CD4+ TCR transgenic mouse model which generates T cells specific for the melanoma antigen TRP-1 (Tyrp1; Muranski et al., 2008). TRP-1 is expressed in B16 melanoma cells, and TRP-1+ T cells are efficacious against established B16 melanoma tumors (Muranski et al., 2008). Importantly, mTECs endogenously express TRP-1 in an Aire–dependent manner, such that TRP-1–specific T cells are deleted in the thymi of Aire–sufficient, TRP-1–sufficient hosts (Muranski et al., 2008; Zhu et al., 2013). Given the deletion of Aire+ mTECs and loss of thymic TRP-1 expression in anti-RANKL–treated mice (Fig. 1 D), we hypothesized that anti-RANKL treatment could rescue TRP-1+ T cells from thymic deletion. We treated RAG1−/− x TRP-1 TCR transgenic mice with either anti-RANKL or control antibody and harvested their thymi and spleens for analysis. Consistent with previous reports, isotype-treated mice showed efficient deletion of CD4 SP T cells in the thymus (Fig. 5 A; Muranski et al., 2008; Zhu et al., 2013). In contrast, anti-RANKL treatment prevented deletion of CD4 SP cells and also resulted in a much higher percentage of Vβ14+ T cells (Fig. 5, A and B). Furthermore, TRP-1+ T cells were detectable in the spleens of anti-RANKL–treated mice and also expressed higher levels of the Vβ14 TCR (Fig. 5, A and B). In addition, qPCR analysis revealed a loss of Tyrosinase and Tyrp1 expression in the ear skin of anti-RANKL–treated mice which appeared consistent with the destruction of melanocytes by TRP-1+ T cells (Fig. 5 C).

Next, we challenged anti-RANKL–treated RAG1−/− x TRP-1 mice with B16 melanoma to determine whether a limited break in central tolerance could improve overall survival. We observed a statistically significant increase in the survival of anti-RANKL–treated mice compared with the isotype–treated cohort (Fig. 5 D). We also found evidence of an enhanced T cell response in anti-RANKL–treated mice by measuring CD3+ T cell infiltrates within the tumors of these mice (Fig. 5 E). Of note, given the overall increase in T reg cells in the spleen and tumors of anti-RANKL–treated mice, it appears unlikely that the survival benefit observed in TRP-1 mice is due to differences in T reg cell numbers (Fig. 5, B and F). Furthermore, to exclude potential effects of anti-RANKL antibody on the tumor microenvironment, we performed adoptive transfer studies in which splenocytes from antibody-treated RAG1−/− x TRP-1 mice were transferred into RAG1−/− mice. When challenged with B16 melanoma, recipients of splenocytes from anti-RANKL–treated donors again showed a statistically significant increase in survival (Fig. 5 G). Collectively, these data demonstrate that short-term, reversible RANKL blockade of mTEC development can be used to create a therapeutic window that allows tumor self-antigen–specific T cells to escape thymic deletion. Furthermore, the generation of these high-affinity tumor-specific T cells confers a survival benefit in a melanoma tumor model.

In conclusion, our findings provide strong evidence that mTECs can be selectively and therapeutically targeted by RANKL blockade in adult mice and that anti-RANKL can be used as an approach to enhance anti-tumor responses. To date, previous efforts on the therapeutic manipulation of TECs have shown global effects that involve both cTECs and mTECs and disrupt thymocyte development (Fletcher et al., 2009). The selectivity for mTECs with anti-RANKL thus provides evidence that central tolerance can be transiently suppressed in adult hosts while maintaining T cell generation. Although autoimmunity is a dangerous potential consequence of this approach, the treatment may also be an attractive new method to help break tolerance for cancer immunotherapy. Interestingly, although we could detect an expansion of retina-specific T cells in anti-RANKL–treated mice, we did not observe development of spontaneous uveitis (unpublished data), suggesting that this brief window of central tolerance suppression was countered by peripheral tolerance mechanisms such as T reg cells which existed before treatment. Importantly, we find that the medullary epithelial compartment of the thymus recovers after withdrawal of anti-RANKL antibody and, thus, only a transient window of central tolerance suppression occurs. This window may be an attractive feature of this approach, especially if coupled with methods that preferentially expand tumor-specific T cells over pathogenic autoreactive T cells. Currently, there has been intense interest and progress in manipulating peripheral tolerance for immunotherapy (Chen and Mellman, 2013). Given our results, it will also be of interest to determine if a combination of methods that target both central and peripheral tolerance could further enhance anti-tumor immune responses. Finally, it is important to note that our results also have implications for patients in the clinical setting receiving denosumab, a humanized monoclonal antibody that blocks RANKL which is widely used in the treatment of osteoporosis in adults (Cummings et al., 2009). Further study will be needed in such patients regarding their susceptibility to autoimmunity and for other potential defects in central tolerance.

MATERIALS AND METHODS

Mice. C57BL/6, B6.RAG1−/− x Tyrp1+tαx TRP-1 TCR transgenic, and B6. OPG−/− mice were purchased from The Jackson Laboratory. Mice were treated intraperitoneally with 100 μg anti-RANKL (IK22/5) or isotype control (2A3; Bio X Cell) antibody three times per week as stated in the text. B6.OT-II, B6.RIP-mOVA, and B6.1R8P−/− mice were described previously (Anderson et al., 2005; DeVoss et al., 2006). Mice were treated at 3–5 wk of age and harvested at time points indicated in the text. All mice were housed and bred in specific pathogen-free conditions in animal facilities at UCSF or
Statistical analysis. Statistical analysis was performed using Prism 6.0 (GraphPad Software). Mann-Whitney Rank sum testing was performed on tetramer analysis. Student’s t test was performed for TEC and lymphocyte analyses. Log-rank test was performed for Kaplan-Meier survival curves.

We thank T. Metzger, T. LaFlam, M. Cheng, and W. Purtha for critical reading of the manuscript. We thank the National Institutes of Health Tetraper Core Facility for providing tetramer reagents.

This work was supported by the US National Institutes of Health Grants AI097457 (M.S. Anderson) and K24-GM081266 (M.L. Mouchess), and the UCSF Medical Scientist Training Program (I.S. Khan). Flow cytometry data were generated in the UCSF Parnassus Flow Cytometry Core, which is supported by the Diabetes and Endocrinology Research Center (DERO) grant NIH P30 DK063720.

The authors declare no competing financial interests.
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