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Leveraging TCR affinity in adoptive immunotherapy against shared tumor/self antigens

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Abstract

Adoptive cellular therapy (ACT) using T-cell receptor (TCR)-engineered lymphocytes holds promise for eradication of disseminated tumors, but also an inherent risk of pathologic autoimmunity if targeted antigens or antigenic mimics are expressed by normal tissues. We evaluated whether modulating TCR affinity could allow $CD8^+$ T cells to control tumor outgrowth without inducing concomitant autoimmunity in a preclinical murine model of ACT. RIP-mOVA mice express a membrane-bound form of chicken ovalbumin (mOVA) as a self-antigen in kidney and pancreas. Such mice were implanted with OVA-expressing ID8 ovarian carcinoma cells and subsequently treated with $CD8^+$ T lymphocytes (CTLs) expressing either a high-affinity (OT-I) or low-affinity (OT-3) OVA-specific TCR. The effects on tumor growth versus organ-specific autoimmunity were subsequently monitored. High-affinity OT-I CTLs underwent activation and proliferation in both tumor-draining and pancreatic lymph nodes, leading to both rapid eradication of ID8-OVA tumors and autoimmune diabetes in all treated mice. Remarkably, the low-affinity OT-3 T cells were only activated by tumor-derived antigen and mediated transient regression of ID8-OVA tumors without concomitant autoimmunity. The OT-3 cells eventually upregulated inhibitory receptors PD-1, TIM-3, and LAG-3 and became functionally unresponsive, however, allowing the tumors in treated mice to reestablish progressive growth. Antibody-mediated blockade of the inhibitory receptors prevented exhaustion and allowed tumor clearance, but these mice also developed autoimmune diabetes. The findings reveal that low-affinity TCRs can mediate tumor regression and that functional avidity can discriminate between tumor-derived and endogenous antigen, while highlighting the risks involved in immune checkpoint blockade on endogenous self-reactive T cells.

Keywords

Adoptive immunotherapy; TCR affinity; Autoimmunity; Immune Checkpoint Blockade; Preclinical animal model

INTRODUCTION

Adoptive T-cell therapy (ACT) is a potent and flexible cancer treatment modality that can induce complete and durable regression of solid tumors.[1, 2] Current cell therapy options include modifying autologous T cells to express either tumor-specific T-cell receptors (TCRs) or chimeric antigen receptors (CARs), which target surface proteins via a single-chain molecule containing an antigen-binding domain fused to transmembrane and cytoplasmic domains required for surface expression and T cell triggering [3, 4]. Although target recognition by CARs occurs in a HLA-unrestricted manner, TCRs have the ability to recognize peptides derived from exogenous, membrane-bound, and intracellular proteins, thus allowing a far greater repertoire of antigens to be targeted. Pioneering studies by Rosenberg and colleagues have demonstrated that ACT with tumor-infiltrating lymphocytes (TILs) can be effective against a variety of solid malignancies including metastatic melanoma, gastrointestinal, and human papillomavirus–induced cancers, and this extends to bulky invasive tumors at multiple sites involving liver, lung, soft tissue, and brain[1, 5–8]. These responses are durable in approximately 20% of treated patients who experience prolonged remissions of five years or more. However, a major limitation to the widespread application of TIL therapy is the difficulty in routinely generating human T cells with antitumor activity outside of a limited number of highly-specialized laboratories.

An alternative approach to TIL-based ACT is the use of high-affinity TCRs against tumor-associated antigens (TAAs) that can be expressed in a patient's normal T cells prior to expansion and reinfusion.[9–13] Such genetically modified T cells have the potential to overcome the variability in the specificity, potency, and longevity of TIL. A major limitation of adoptive cell therapy utilizing such genetically modified cytotoxic T lymphocytes (CTLs) has been "on-target, off-tumor" toxicities associated when the transferred cells recognize antigens expressed on both tumor cells and normal tissue.[14–17] In the first clinical trial using such engineered T cells targeting the melanoma TAA MART-1, objective responses were seen in 30% of melanoma patients receiving MART-1–targeted T cells, although over 80% of patients (29/36) developed an erythematous skin rash, and others developed anterior uveitis or hearing loss attributable to recognition of low expression of MART-1 in normal melanocytes, retina and inner ear.[9] Similarly, a phase I trial using genetically modified T cells targeting carcinoembryonic antigen (CEA) resulted in severe transient inflammatory colitis as a result of "on-target" effects of the engineered T cells against colonic tissue expressing CEA.[15] Another trial with genetically modified T cells targeting the cancer testis antigen (CTA) MAGE-A3, known to be expressed in a range of epithelial malignancies but not most normal tissues, resulted in severe neurologic toxicity from periventricular infiltration of adoptively transferred $CD3^+$ $CD8^+$ T cells with resultant necrotizing leukoencephalopathy and death in 2 of 9 treated patients due to previously unrecognized expression of MAGE antigens in the human brain.[14]

To better understand the risks involved in ACT, we developed a new preclinical model to study the phenomenon of "on-target, off-tumor" toxicities in the context of ACT, which features adoptively transferred T cells specific for an antigen expressed on both tumor and normal tissue. This system is based on a well-characterized murine model (RIP-mOVA), which features steady-state surface expression of a model antigen (membrane-bound chicken

ovalbumin or mOVA) on proximal tubular cells of the kidney and beta islets of the pancreas. Although these mice lack high-affinity OVA-specific $CD8^+$ T cells in their peripheral repertoire due to central and peripheral tolerance mechanisms, adoptive transfer of high-affinity OT-I cells specific for the $OVA_{257-264}$/H-$2K^b$ complex rapidly leads to their activation with subsequent destruction of beta cells, and autoimmune diabetes. RIP-mOVA mice do possess a low-affinity OVA-specific T cell repertoire, which can be elicited by vigorous vaccination and can occasionally cause autoimmune diabetes (18). OT-3 mice are transgenic for one such low-affinity $OVA_{257-264}/K^b$–specific TCR that was isolated from immunized RIP-mOVA mice [18]. In contrast to OT-I, OT-3 T cells become neither activated nor anergic in response to mOVA expression in pancreas or kidney tubulointerstitium following adoptive transfer to RIP-mOVA mice, and instead maintain their potential for response. In this way, OT-3 T cells are functionally analogous to the post-thymic repertoire of self-reactive T cells that exists in the periphery of healthy human subjects. This model is therefore distinct from previous examples in which low-affinity T cells against a shared tumor/self antigen are rendered profoundly anergic by expression of the target antigen on self-tissues [19].

Our objective in these studies was to determine whether TCR affinity could discriminate between self and tumor tissues expressing the same antigen. To address this, we established tumors in RIP-mOVA mice using an ovarian cancer model modified to express membrane-bound OVA (ID8-mOVA), and monitored tumor control versus autoimmune diabetes development following ACT with OT-I or OT-3 $CD8^+$ T cells (Supplementary Fig. S1). We found that the low-affinity OT-3 T cells could be cross-primed and activated by antigen originating from the tumor, but not from normal self-tissues, and that these cells could thence mediate the selective eradication of tumor cells without concomitant autoimmune beta cell destruction. OT-3 T cells eventually became functionally unresponsive, and treatment with checkpoint blockade immunotherapy both restored their functionality with respect to tumor control, but also led to diabetes induction. These findings demonstrate that TCR affinity can allow ACT to discriminate between tumor and self, while highlighting the risks that exist for the use of systemic immune checkpoint blockade immunotherapy.

MATERIALS AND METHODS

Mice

Mice were maintained under specific pathogen-free conditions in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. The studies described in this paper conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. C57BL/6J (B6; $CD45.2^+$), B6.SJL ($CD45.1^+$), RIP-mOVA and *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratory. Ovalbumin (OVA)-specific OT-I TCR-transgenic (Tg) $CD45.1^+$ and Ovalbumin (OVA)-specific OT-3 mice on a B6 background have been previously described. Six to 12 week-old mice were used in all experiments.

Cell lines

ID8-OVA cells were a kind gift from Dr. Dietmar Zehn (Technical University Munich) in 2014. The B3Z T cell hybridoma was a kind gift from Dr. Nilabh Shastri (University of California, Berkley). All tumor cell lines were tested prior to *in vivo* use and found to be free of mycoplasma. In addition model antigen expression for OVA was confirmed by flow cytometry. Due to the potential for cross-contamination of cell lines, ID8-OVA cells were re-authenticated by flow cytometry at least every three months and cells were maintained in culture for no more than 30 passages. Mycoplasma testing was repeated at least every six months.

Flow cytometry, intracellular cytokine staining and cytokine analysis

Single-cell suspensions were prepared from spleens, inguinal lymph nodes, and tumors. Cells were stained with fluorescent-labeled antibodies (BioLegend, San Diego, CA; BD-Bioscience Pharmingen, San Diego, CA; or eBiosciences, San Diego, CA) and analyzed by either FACSCalibur or LSR II flow cytometer (BD, San Diego, CA). The following clones were used: CD4 (RM4-5), CD8 (5H1), CD11c (HL3), Cd11b (M170), CD25 (PC61.5), SIINFEKL/H-2Kb (25-D1.16), CD44 (IM7), CD62L (MEL-14), IFNγ (XMG1.2), TNFα (MP6-XT22), FAS (2495), CD40 (3/23) and Foxp3 (FJK-16s). For intracellular cytokine staining, cells were activated with OVA peptide-pulsed splenocytes or with PMA (100 ng/ml) plus ionomycin (500 ng/ml) for 4 hours in the presence of GolgiPlugTM (BD Biosciences, California), processed with a Cytofix/CytopermTM kit (BD Biosciences, California), and stained as indicated. Gates and quadrants were set based on isotype control staining unless otherwise indicated.

Adoptive Transfer Experiments and CTV Labeling

T cells from spleens and lymph nodes (LN) of OT1-Rag KO and OT-3 TCRalpha KO mice were used for adoptive cell transfer experiments. $CD8^+$ T cells were isolated by negative selection of single cell suspensions from homogenized donor tissue using a MACS CD8 isolation kit (Miltenyi Biotec, Germany). Purity of isolated $CD8^+$ T cells was confirmed by flow cytometry analysis and was routinely greater than 95% with less than 0.1% $CD4^+$ T cell contamination. Donor T cells were obtained from CD45.1 mice, whereas recipient mice were CD45.2, thereby permitting *in vivo* isolation via this congenic marker. For *in vivo* cell proliferation studies, T cells were washed twice in PBS and labeled in 5nM CTV (25×10^6 cells/ml) for 8 minutes at 37°C on a shaker followed by quenching with ice-cold media. Cells were washed twice in PBS, and 5×10^6 cells were injected per mouse via retro-orbital injection. Mice were sacrificed 3 days later, and the spleen and lymph node cell suspensions were analyzed via flow cytometry. For other adoptive transfer experiments donor T cells were harvested from the draining lymph node and spleens at the indicated time points for analysis.

Ectopic ID8 tumor model, Intravital Imaging (IVIS) and tumor vaccinations.

Previously published methods were used for ectopic implantation of ID8 tumor cells.[20] Briefly, while anesthetized, mice were inoculated S.C. with single cell suspensions of 5×10^6 tumor cells suspended in 200 μL of PBS into the right flank. Three weeks later, the mice

were monitored for tumor growth by tumor bioluminescence using an IVIS system and in response to treatment thereafter at the indicated time points. To monitor for tumor bioluminescence, mice received 300 μg of D-luciferin (Promega) by intraperitoneal injection 10 minutes before imaging ID8-OVA-luc tumors using a Xenogen IVIS 100 to maximum tumor radiance. Results are reported as the percent change from baseline tumor radiance on the day of treatment. For tumor vaccination studies, single cell suspensions of tumor cells (10^7/mL) in PBS were subjected to 150 Gy of gamma ionizing radiation using an irradiator prior to S.C. flank inoculations of 5×10^6 cells into the right flank of recipient mice.

Antigen-specific immune response assay

Spleen and LN cells were incubated for 5 hours with the $OVA_{257-264}$ (SIINFEKL) at 5 μg/mL final concentration in the presence of Brefeldin A (BD Biosciences, California) directly *ex vivo* and were stained for surface expression of CD8 and CD3, fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences, California) and stained for intracellular IFNγ according to manufacturer's protocol.

B3Z assays

B3Z is a *lacZ*-inducible $CD8^+$ T cell hybridoma expressing TCR specific for $OVA_{257-264}$ (SIINFEKL), presented on the murine $H2K^b$ MHC class I molecule [21]. ID8-OVA-luc, SAMBOK or MECI cells were cocultured with B3Z for 3 hours at 37°C in a 5% CO_2/air atmosphere, after which cells were washed, and β-galactosidase activity was detected by addition of substrate (CPRG, Sigma-Aldrich) and absorption readings at 600 nm using a spectrophotometer plate reader.

Monoclonal antibodies for in vivo immune checkpoint studies

The following mAbs were purchased from BioXcell: InVivoPlus anti-mouse PD-1 (J43; Cat. #BP0033-2), InVivoPlus anti-mouse LAG-3 (C9B7W; Cat. #BP0174) and InVivoPlus anti-mouse TIM-3 (RMT3-23; Cat. #BP0115). A total of 250 μg of each antibody was transferred intravenously in 200 μL of PBS into recipient mice.

Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad, La Jolla, CA). One-way ANOVA and unpaired two-tailed Student *t* tests were conducted and considered statistically significant at *p* values ≤ 0.05 (*), 0.01 (**) and 0.001 (***).

RESULTS

Induction of autoimmune diabetes by OT-3 CTLs.

We first sought to determine to determine the quantitative threshold for diabetes induction by fully activated OT-3 in RIP-mOVA mice in the setting of ACT. Naïve OT-3 $CD8^+$ T cells were harvested from donor mice by negative selection of homogenized splenocytes, and graded doses (10^3, 10^5 or 10^6) were transferred to recipient RIP-mOVA mice, which were subsequently infected by an OVA-expressing strain of wildtype *Listeria monocytogenes* (Lm-OVA) by intravenous injection. Mice were then monitored for diabetes induction (blood

glucose > 200 mg/dL) daily after transfer. Despite the lower functional avidity for the OVA antigen expressed by the pancreatic beta cells, systemic activation of 10^6 OT-3 cells by Lm-OVA resulted in autoimmune destruction of beta islets in all RIP-mOVA mice (Fig. 1A). In addition, 10^6 OT-3 cells were also activated by an attenuated strain of Lm-OVA ($ActA^-$) that is unable to spread laterally via actin polymerization, demonstrating that a single priming stimulus, and not continued activation by proliferating Lm-OVA within reticuloendothelial cells, is sufficient to result in autoimmune diabetes (Fig. 1B). Finally, this phenomenon was dose-dependent, requiring 10^5 OT-3 cells to develop diabetes at 100% penetrance of the phenotype (Fig. 1C). These data indicate that OT-3 cells can be activated and induce autoimmunity in RIP-mOVA mice reproducibly with the appropriate activating stimulus.

ID8-OVA tumors induce proliferation of adoptively transferred OT-3 CTLs via cross presentation.

Although OT-3 cells can induce autoimmune diabetes in RIP-mOvA after priming with a strong stimulus like Lm-OVA infection, it was not known if a progressively-growing tumor could also do so. To address this, OT-3 cells were labeled with the viable dye CTV (which allows visualization of cellular division by its portioning into daughter cells) and adoptively transferred to RIP-mOVA harboring ID8-OVA tumors. The mice were euthanized 96-hours following transfer, and the transferred cells were analyzed within the tumor-draining lymph node for proliferation using their congenic marker (CD45.1). The OT-3 T cells within RIP-mOVA mice bearing ID8-OVA tumors both proliferated (Fig. 2A) and acquired effector functionality (Fig. 2B), whereas those transferred to RIP-mOVA mice lacking tumors did neither. These results indicate that naive OT-3 cells can be activated to proliferate and by cross-presentation of OVA antigen in the tumor draining lymph node.

OT-I and OT-3 CTLs have different thresholds of antitumor reactivity versus autoimmune diabetes

Having established that OT-3 T cells cause autoimmune diabetes when systemically activated by a strong inflammatory stimulus (Lm-OVA), and that ID8-OVA tumors engraft in RIP-mOVA mice and are able to cross-prime adoptively transferred OT-3 T cells, we sought to compare whether OT-3 versus OT-I CD8 T cells could mediate control of ID8-OVA tumors without simultaneous off-tumor, on-target autoimmune destruction of pancreatic beta islets. To address this, 5×10^6 OT-I or OT-3 CD8 T cells were transferred to ID8-OVA tumor-bearing RIP-mOVA mice, which were then monitored simultaneously for changes in tumor size by CCD and diabetes induction by blood glucose analysis. As shown in Fig. 3, although the high affinity OT-I T cells were able to mediate destruction of ID8-OVA tumors, they did so at the expense of autoimmune destruction of the beta islets, as demonstrated by development of diabetes (Fig. 3A). OT-3 cells, in contrast, were able to achieve partial destruction of the incipient tumor, but without concomitant autoimmunity. This effect was transient, however, and despite the partial destruction of the ID8 tumor by OT-3 ACT, the tumors eventually resumed progressive growth in all treated mice, suggesting that the transferred T cells had become ineffective or functionally silenced (Fig. 3B).

OT-3 CTLs upregulate inhibitory receptors in response to ID8-OVA tumors and become exhausted

We next investigated the functional status of OT-3 T cells from mice whose tumors had reinitiated progressive growth. To accomplish this, OT-3 T cells were isolated from tumor-draining lymph nodes of treated mice and tested for their ability to produce effector cytokines in response to restimulation by their cognate peptide (SIINFEKL) alongside those primed by the same ID8-OVA tumor offered as an irradiated cellular vaccine. As shown in Fig. 4, whereas the OT-3 T cells were clearly detectable in the tumor-draining lymph node, they were unable to produce IFNγ in response $OVA_{257-264}$ (SIINFEKL) peptide, although those primed by the ID8 vaccine were able to do so. The OT-3 cells from tumor-bearing mice were nonetheless able to produce IFNγ following treatment with PMA/Ionomycin, indicating that the functional defect was in TCR-mediated activation (Fig. 4A). These findings demonstrate that the growing tumor is able to induce a state of unresponsiveness, termed “exhaustion”, in the responding OT-3 T cells. Accordingly, the OT-3 cells adoptively transferred to mice bearing ID8-OVA tumors versus ID8-WT tumors, or mice vaccinated with irradiated ID8-OVA cells, demonstrated up-regulation of the inhibitory coreceptors PD-1, Tim-3 and Lag-3 (Fig. 4B).

Immune checkpoint inhibitors reverse OT-3 CTL exhaustion but induce autoimmune diabetes

Inhibitory receptors on immune cells are pivotal regulators of immune escape in cancer [22]. We have shown that adoptively transferred OT-3 cells demonstrate extensive coexpression of PD-1, LAG-3, and TIM-3, and we have documented functional exhaustion in SIINFEKL antigen restimulation experiments following adoptive cell transfer into RIP-mOVA mice bearing ID8-Ova tumors. T-cell exhaustion is a plausible mechanism for the transient tumor control elicited by OT-3 cells in recipient mice. We therefore next sought to determine if co-administration of immune checkpoint inhibitors along with OT-3 cells could preclude exhaustion and potentiate the antitumor reactivity of the adoptively transferred T cells in RIP-mOVA mice bearing ID8-OVA tumors. In these experiments, 250 μg of anti-PD1, anti-Lag3, anti-Tim3, or a combination of all three immune checkpoint antibodies were administered to the contralateral orbital sinus along with adoptive cell transfer of 5×10^6 OT-3 cell into ID8-OVA tumor-bearing RIP-mOVA mice. Co-administration of immune checkpoint inhibitors did not demonstrate statistically significant changes in tumor killing versus controls (Fig. 5A). However, linear regression analysis of all treated animals from all groups demonstrated that tumor killing did correlate with blood glucose concentration in treated mice (Fig. 5B). The highest blood glucose and tumor killing responses were all observed in the combinatorial immune checkpoint cohort that received OT-3 cells in addition to PD-1, Lag-3 and Tim-3 inhibitors. In this group, half of the treated mice developed autoimmune diabetes with a trend towards greater tumor killing (Fig. 6). In addition, OVA antigen restimulation of splenocytes harvested from treated mice showed higher responses in the diabetic vs. nondiabetic mice in this group. These data suggest that combinatorial ICB can preclude functional exhaustion in adoptively transferred OT-3 cells with a trend toward enhanced tumor killing but at the expense of developing autoimmune diabetes.

DISCUSSION

The current landscape of clinical cancer immunotherapy involves strategies aimed at both uncoupling a patient's T cells from negative regulation as well as the adoptive transfer of those which have been expanded *in vitro* and in some settings, engineered to acquire antitumor specificity [23–25]. In each of these settings, the risk of pathologic autoimmunity must be balanced with the therapeutic potential inherent in trying to direct powerful effectors exclusively towards the tumor and its antigens. The model system established and described in this work represents an effort to explore these dichotomies in the context of varying TCR affinity in a controlled preclinical setting to better understand their causes, and thereby, to inform clinical practice.

Our results clearly show that T cells possessing an antigen receptor of sufficiently low functional avidity against a normal self-antigen to escape central tolerance can nonetheless be activated when the antigen in question is expressed on a growing tumor. In many key aspects, the OT-3 T cells in RIP-mOVA mice can be considered as a surrogate for the post-thymic selection peripheral repertoire of potentially self-reactive T cells which exist in a state of 'immunological ignorance' with respect to their cognate antigen, undergoing neither activation nor tolerance until some crucial aspect of their regulation is disrupted to enable their full activation. In the present study, this transition could be (unsurprisingly) induced by a strong inflammatory challenge with an OVA-expressing bacterial pathogen, or by a progressively growing tumor, with the latter leading to both proliferation and acquisition of effector functionality, producing a transient reduction in tumor volume. Remarkably, activation of the OT-3 T cells under these conditions did not lead to autoimmune diabetes, despite OVA expression on beta cells, suggesting that a window of opportunity exists for the selective targeting of a tumor by self-reactive T cells without concomitant recognition of a self tissue sharing expression of the same antigen. These results do not exclude clinically subapparent toxicity, since elevated serum glucose concentration is typically detectable once 90% of insulin-producing cells are destroyed. The basis for this selective recognition of tumor over self is unclear, but could result from differences in the level of antigen presented by the two target cell populations or the local regulatory environment which may govern access and functional recognition of the OVA antigen in two sites. It is worth noting that, in light of the previously-stated ignorance of OVA as an antigen by the OT-3 cells, the $OVA_{257\text{-}264}/K^b$ epitope is functionally equivalent to a foreign antigen for the OT-3 T cells, albeit one they recognize with low affinity. In the context of their selective recognition of the ID8-OVA tumor cells, $OVA_{257\text{-}264}/K^b$ functions as a tumor-specific neoantigen. In this regard, our findings suggest that such low-affinity T cells, whether induced by vaccination or provided through adoptive transfer, can be an effective therapeutic force for eradicating established tumors

Although the low-affinity OT-3 T cells could be selectively activated by tumor-derived OVA and mediate some degree of tumor killing, the effect was temporary and the population eventually underwent functional silencing accompanied by coordinated upregulation of immune checkpoint molecules PD-1, TIM-3, and LAG-3. In this regard, the low-affinity T cells behaved as numerous reports have demonstrated for the endogenous tumor-reactive T cell repertoire [26]. Attempts to overcome this "exhausted" state through antibody-mediated

blockade of the relevant receptors restored antigen-specific production of the effector cytokine IFNγ, by OT-3 T cells, but the ensuing response invariably led to diabetes induction in the majority of treated animals in the current study. These findings demonstrate that this model system can allow an unprecedented degree of insight into the hazards implicit in systemic blockade of immune checkpoint pathways when a self-reactive T cell population has been activated, with autoimmune diabetes being the primary immune-mediated adverse event (irAE) observed [27].

A number of studies have demonstrated the existence of optimal TCR “affinity windows” for key parameters of T cell function including proliferation, antigen sensitivity, and polyfunctionality [28], above which signaling can render cells hyporesponsive to low amount of antigen and/or mediate dangerous cross-reactivity to self MHC [29–31]. These findings add to the growing consensus that TCRs with low to moderate affinities may possess important functional features necessary for sustained signaling to physiological concentrations of antigen [32, 33]. This notion is supported by studies by Sherman and colleagues, who showed that vaccination against an antigen shared between tumor and self tissue can induce low avidity antigen-specific $CD8^+$ T cells to reject tumor cells with high expression of target antigen yet remain tolerant of antigen-expressing pancreatic beta cells. [34] In adoptive cell transfer experiments conducted by this group, low avidity antigen-specific T cells that were tolerized by cross-presented tumor antigen could subsequently eradicate tumors if antigen-specific $CD4^+$ T cells were cotransferred into tumor bearing mice harboring the target antigen, lending further evidence that such “affinity tuned” cytotoxic T cells can be exploited therapeutically.[35, 36]

This study reports that under conditions of steady-state progressive growth, a low-affinity TCR can preferentially be primed to and recognize antigen on tumor without causing autoimmune pathology to a normal self-tissue sharing its expression, whereas a high-affinity TCR was unable to discriminate between the two types of target cells. This suggests that, whether due to the amount of antigen produced or the homeostatic state of the tissue in question, the tumor environment is distinct from that of a stable self-tissue in terms of the magnitude and context of antigen (cross-)presentation and that only a low-affinity TCR was capable of responding to this. This finding suggests that further investigation of low-affinity TCRs could further elucidate the factors that govern the functionality in the setting of ACT against shared tumor/self antigens and in doing so augment the precision and efficacy of ACT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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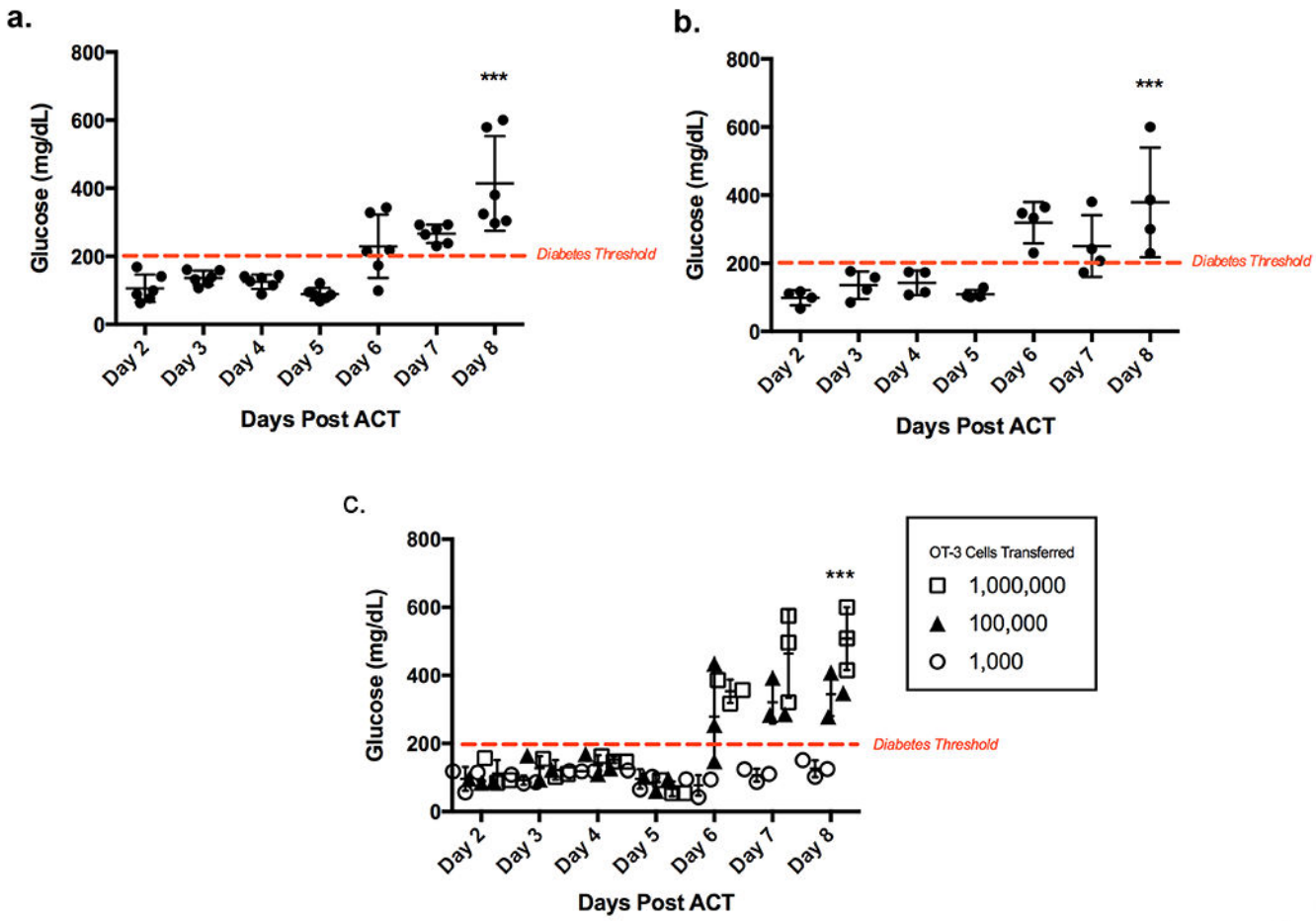

Fig. 1. Induction of autoimmune diabetes by OT-3 cytotoxic T lymphocytes.

A) Systemic activation of 10^6 adoptively transferred OT-3 $CD8^+$ T cells by intravenous (i.v.) cotransfer of 3,000 CFU of OVA-expressing Listeria monocytogenes (Lm-OVA) resulted in autoimmune destruction of beta islets in RIP-mOVA mice. B) Systemic activation of OT-3 CD8 T cells by intravenous (i.v.) cotransfer of 10^6 CFU of Lm-OVA deficient for Act A (Lm-OVA Act A−/−) is sufficient to result in autoimmune diabetes. C) Autoimmune diabetes induced by adoptively transferred OT-3 $CD8^+$ T cotransferred with 3,000 CFU of Lm-OVA cells is dose-dependent, requiring 10^5 OT-3 T cells to develop diabetes at 100% penetrance of the phenotype.

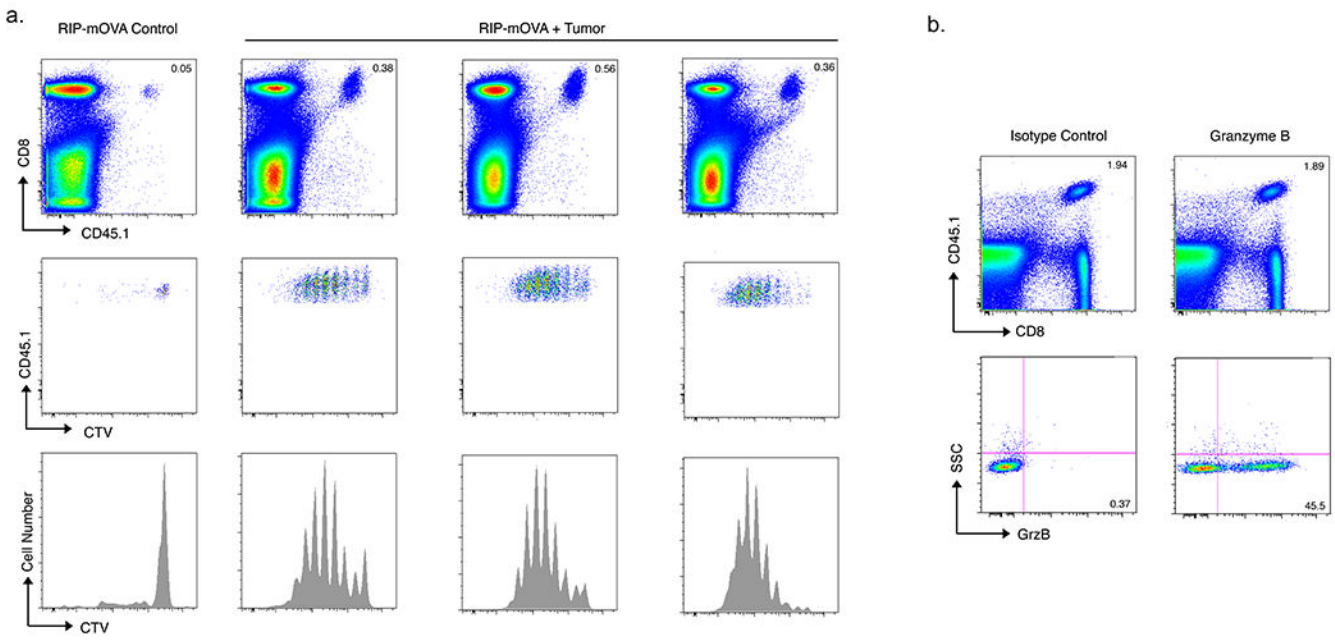

Fig. 2. ID8-OVA tumors induce proliferation of adoptively transferred OT-3 CD8 T cells by cross presentation.

A) RIP-mOVA mice bearing 3-week old ID8-OVA tumors elicited proliferation of adoptively transferred OT-3 $CD8^+$ T cells that were identifiable by the congenic marker CD45.1 and labeled with cell-trace violet (CTV) within the tumor draining lymph node 72 hours post-transfer, whereas mice lacking tumors did not demonstrate proliferation. B) Adoptively transferred OT-3 $CD8^+$ T cells into tumor bearing mice produced Granzyme-B 72 hours post-transfer, consistent with functional activation.

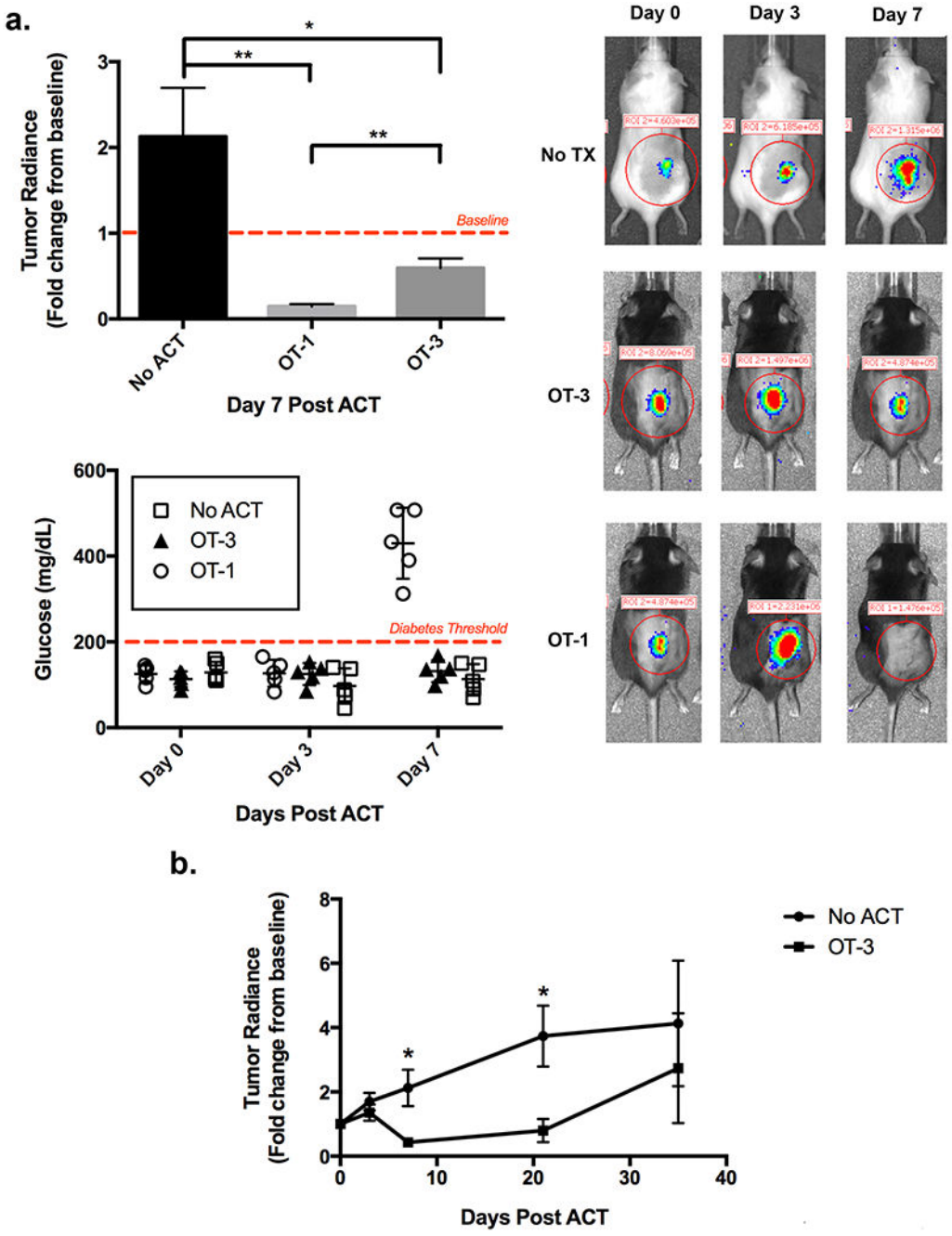

Fig. 3. OT-1 and OT-3 CD8 T cells have different thresholds of anti-tumor reactivity versus autoimmune diabetes.

A) Adoptive cell transfer of 5×10^6 naïve OT-I $CD8^+$ T cells, prepared by negative selection from donor transgenic OT-I mice splenocytes and lymph nodes, mediates destruction of ID8-OVA tumors as reflected by decreased tumor bioluminescence. However, all recipient mice develop autoimmune diabetes. ACT of OT-3 $CD8^+$ T cells, in contrast, mediates partial destruction of the incipient tumor without concomitant autoimmunity. B) ID8-OVA tumors that are transiently controlled by ACT of OT-3 $CD8^+$ T cells demonstrate progressive tumor outgrowth by 21 days post cell transfer.

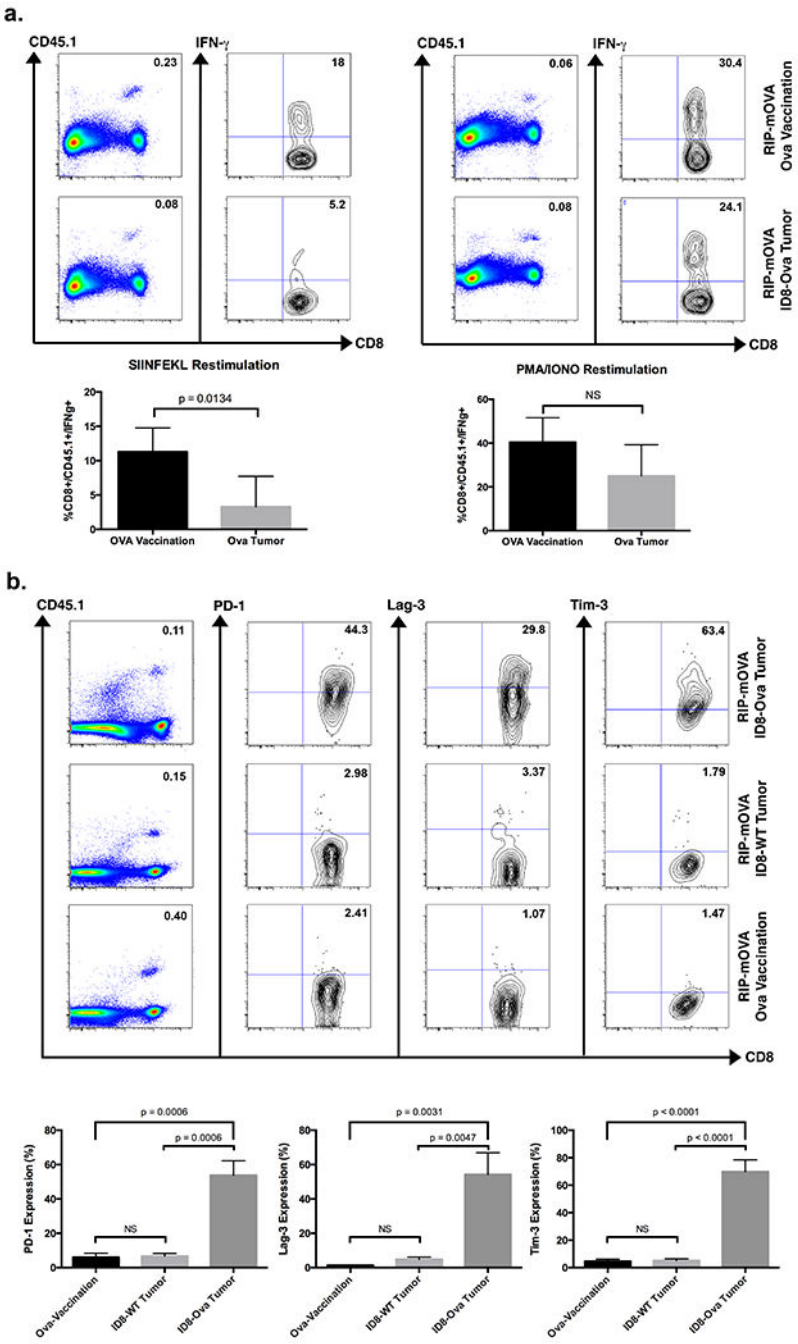

Fig. 4. OT-3 CD8 T cells upregulate inhibitory receptors in response to ID8-OVA tumors and become functionally exhausted.

A) 5×10^6 OT-3 $CD8^+$ T cells adoptively transferred to mice bearing mature ID8-OVA tumors and harvested from the tumor draining lymph node 15 days post-transfer are unable to produce IFNγ in response OVA257-264 (SIINFEKL) peptide restimulation, whereas OT-3 $CD8^+$ T cells primed by an ID8 tumor cell vaccine that lacks persistence of the tumor cells *in vivo* demonstrate successfully SIINFEKL antigen-specific restimulation. B) OT-3 $CD8^+$ T cells adoptively transferred to mice bearing ID8-OVA tumors and harvested from the tumor draining lymph node 15 days post-transfer demonstrate upregulation of the

inhibitory coreceptors PD-1, Tim-3, and Lag-3, whereas mice bearing ID8-WT tumors or mice vaccinated with irradiated ID8-OVA cells did not upregulate exhaustion markers.

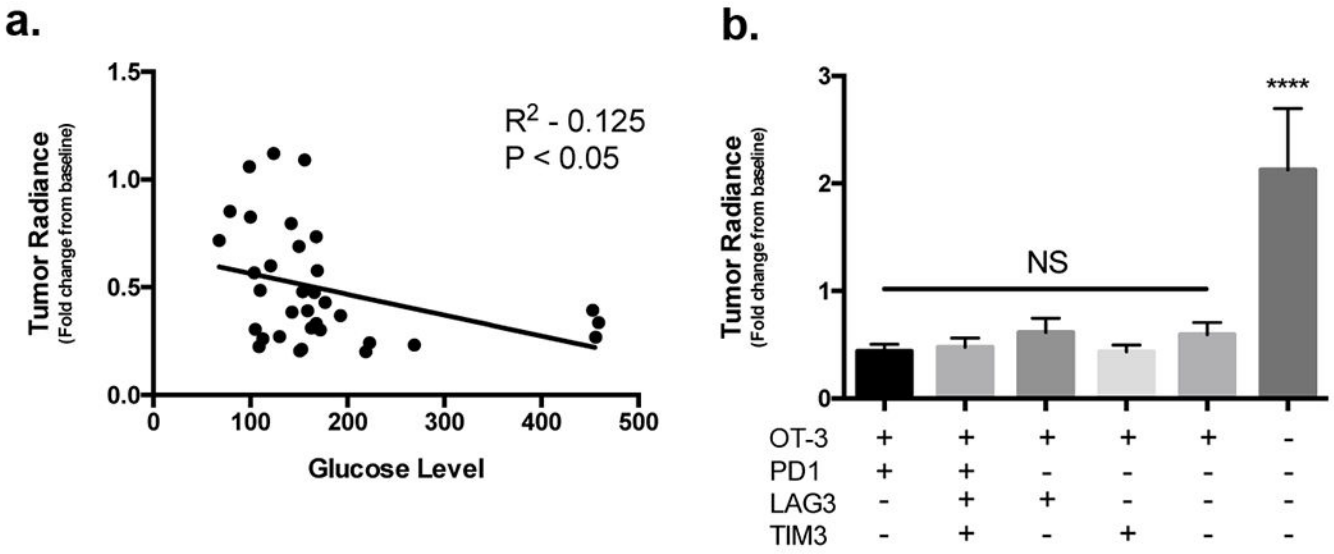

Fig. 5. Cotransfer of OT-3 CD8$^+$ T cells with the immune checkpoint inhibitors anti-PD1, anti-TIM3, and anti-LAG3 elicits autoimmune diabetes that correlates with antitumor responses.

A) Intravenous co-administration of the immune checkpoint inhibitors anti-PD1, anti-TIM3, and anti-LAG3 (250 μg) with 5×10^6 OT-3 CD8$^+$ T cells into RIP-mOVA mice bearing 3-week old ID8-OVA tumors did not demonstrate statistically significant changes in tumor killing by tumor bioluminescence by day 7 post-ACT as single agents or in combination vs. mice that only received OT-3 CD8$^+$ T cells B) Linear regression analysis of all treatment conditions did demonstrate that tumor killing correlates with blood glucose concentration in treated mice.

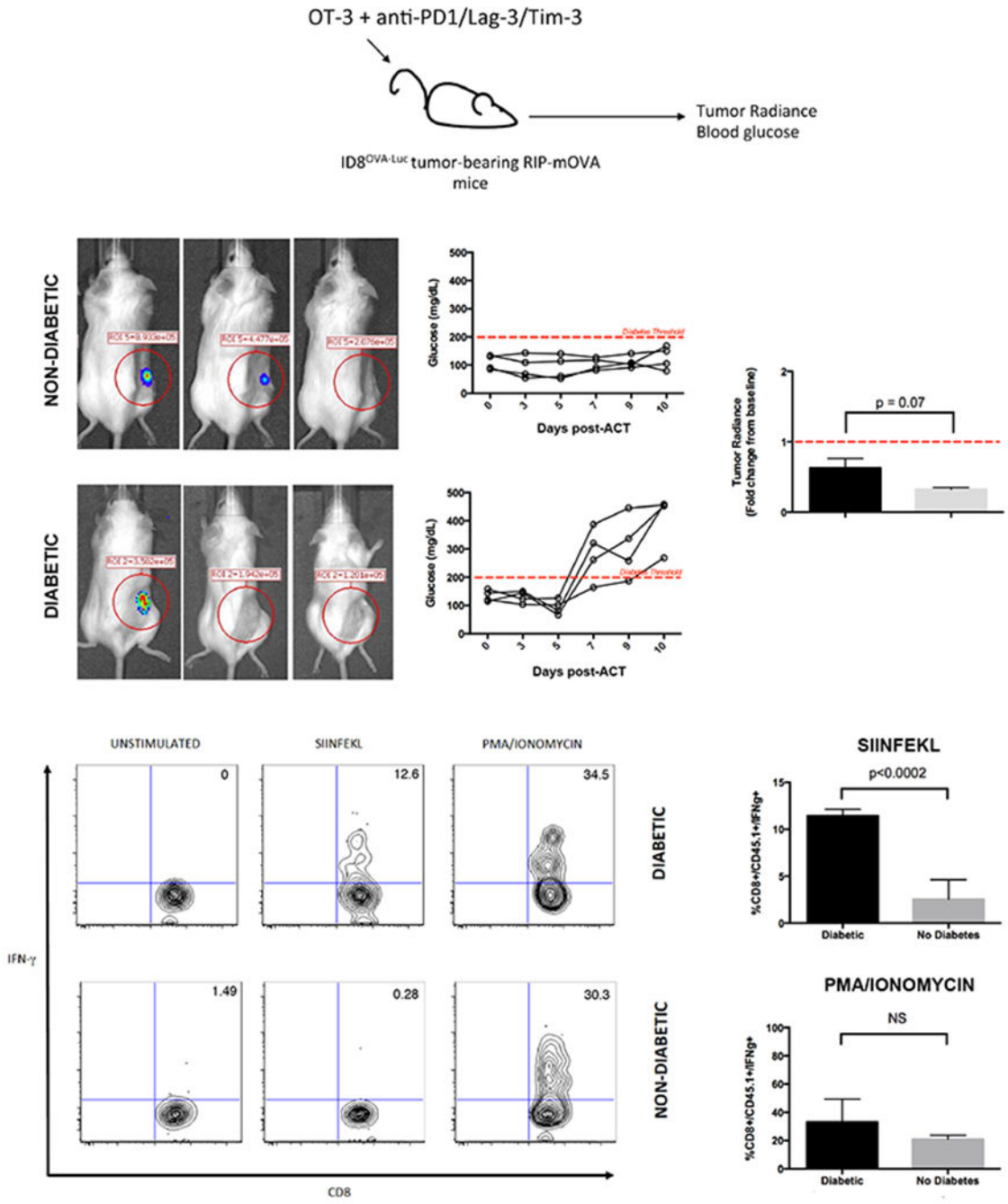

Fig. 6. Combinatorial immune checkpoint blockade with anti-PD1, anti-TIM3, and anti-LAG3 reverses OT-3 CD8$^+$ T cell exhaustion with a trend to enhanced tumor killing but induces autoimmune diabetes.

A) Intravenous co-administration of anti-PD1, anti-TIM3, and anti-LAG3 (250 μg) with 5×10^6 OT-3 CD8$^+$ T cells into RIP-mOVA mice bearing 3-week old ID8-OVA tumors induced diabetes in half of the treated mice. B) There is a non-statistically significant trend towards greater tumor killing in mice that developed autoimmune diabetes. SIINFEKL

restimulation of splenocytes harvested from treated mice showed higher responses in the diabetic mice vs. non-diabetic mice in this group.