

Mammary tumors with diverse immunological phenotypes show differing sensitivity to adoptively transferred CD8+ T cells lacking the *Cbl-b* gene

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Abstract We tested the efficacy of CD8+ T cells lacking the *Cbl-b* gene against a panel of mammary tumor lines with different intrinsic sensitivities to T cells. Mice bearing established tumors expressing an ovalbumin-tagged version of HER-2/*neu* underwent adoptive transfer with *Cbl-b*-replete or -null CD8+ T cells from OT-I T cell receptor transgenic donor mice. In general, *Cbl-b*-null OT-I cells showed enhanced expansion, persistence, and capacity for tumor infiltration. This resulted in markedly enhanced efficacy against two tumor lines that normally demonstrate complete (NOP21) or partial (NOP23) regression. Moreover, a third tumor line (NOP6) that normally demonstrates progressive disease underwent complete regression in response to *Cbl-b*-null OT-I cells. However, a fourth tumor

line (NOP18) was resistant to *Cbl-b*-null OT-I cells owing to a profound barrier to lymphocyte infiltration. Thus, *Cbl-b*-null CD8+ T cells are generally more efficacious but are nonetheless unable to mediate curative responses against all tumor phenotypes.

Keywords Breast cancer · Animal models for tumor immunology · Adoptive T cell therapy

Introduction

Adoptive immunotherapy is a promising approach for the treatment of cancer [8, 9, 16, 27]. The strategy involves the isolation and expansion of autologous, tumor-reactive T cells, which are then infused into the patient with the expectation they will recognize cognate tumor antigen and elicit tumor-specific cytotoxicity. While objective clinical responses have been reported in over 50% of patients with advanced melanoma [8, 9], responses are often mixed, wherein some tumor nodules regress while others do not [29]. One barrier to success has been the limited persistence of adoptively transferred T cells in vivo [28, 31, 38, 42]. In addition, barriers in the tumor microenvironment can impede T cell trafficking, infiltration, and effector function at a local level, which likely accounts for the mixed responses seen in many patients [20].

To investigate barriers to successful adoptive immunotherapy, we have developed a novel transgenic mouse model of breast cancer. Briefly, CD4+ and CD8+ T cell epitopes from the model antigen ovalbumin (OVA) were linked to the C terminus of rat *neu* to generate a fusion protein designated NEU^{OT-I/OT-II}. The OVA epitopes are recognized in the context of MHC class I and II by the TCR transgenic mouse strains OT-I (CD8+) and OT-II (CD4+),

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respectively [2, 14]. Expression of *Neu*^{OT-I/OT-II} as a transgene in the mammary epithelium of C57BL/6 mice, together with a dominant negative version of p53, leads to the spontaneous development of mammary adenocarcinomas when mice are 6–10 months of age [32]. Remarkably, adoptive transfer of OT-I and OT-II T cells induces complete regression of approximately 37% of tumors with the remaining tumors showing partial/stable responses (40%) or progressive disease (23%) [32]. Thus, OT-I and OT-II T cells are effective against some but not all tumors in this model, similar to the mixed responses seen in clinical immunotherapy trials, as discussed above. This provides a unique experimental system to evaluate strategies to improve the efficacy of adoptive T cell therapy.

The E3-ubiquitin ligase CBL-B plays a negative regulatory role in T cell activation by marking TCR-related signaling molecules for ubiquitin-mediated degradation. Molecules affected by Cbl-b-mediated regulation include VAV-1, LFA-1, TCR, CRK-L, and the p85 regulatory subunit of PI3-kinase [1, 5, 6, 10, 11, 33, 41]. Accordingly, *Cbl-b*-null T cells show enhanced activity of signaling mediators such as AKT, PLC- γ 1, ERK, PKC- θ , and NF- κ B [23, 24, 26, 30, 33, 40]. Such T cells also show increased TCR clustering and lipid raft aggregation [17], as well as enhanced clustering of LFA-1 and adhesion to ICAM-1 [41]. Upon CD28 costimulation, CBL-B itself is ubiquitinated and degraded, thereby reversing CBL-B-mediated inhibition and facilitating T cell activation [19, 39]. In terms of functional properties, *Cbl-b*-null CD4⁺ and CD8⁺ T cells have a reduced requirement for CD28 co-stimulation with respect to proliferation and production of IL-2 and IFN- γ [1, 4, 5]. Moreover, *Cbl-b*-null T cells are resistant to anergy [15], inhibition by TGF- β [4, 36, 37], suppression by regulatory T cells (Tregs) [21, 36], and functional exhaustion [25]. As might be expected, *Cbl-b*-null mice are prone to experimental and spontaneous autoimmune disease [1, 5, 15].

Since lack of costimulatory molecules in the tumor microenvironment can be a barrier to T cell activity, the use of *Cbl-b*-null T cells has been explored in several tumor models. With respect to immune surveillance, *Cbl-b*-null mice have been shown to develop fewer tumors in the settings of UVB irradiation and ataxia telangiectasia-mutated (ATM) deficiency [4, 21]. Of greater clinical relevance, adoptively transferred *Cbl-b*-null CD8⁺ T cells showed enhanced efficacy against E.G7 lymphomas and TC-1 fibroblast tumors [4, 21] with a reduced dependence on CD4⁺ T cell help [21], suggesting this may represent an attractive treatment strategy for cancer. While these results are promising, it remains to be determined whether the enhanced efficacy of *Cbl-b*-null T cells applies to other tumor types, particularly those displaying the phenotypic diversity evident in human cancer. Given our previous results showing

that spontaneous mammary tumors differ widely in their inherent sensitivity to T cells, we investigated whether *Cbl-b*-null T cells would show universally enhanced efficacy in this model system.

Materials and methods

Mice

This study followed Canadian Council for Animal Care guidelines and was approved by the University of Victoria Animal Care Committee. All mice were C57BL/6 (H-2^b). Mice expressing the *Neu*^{OT-I/OT-II} transgene in mammary epithelium under the control of the MMTV promoter have been described [32], as have TCR transgenic OT-I mice that recognize OVA residues 257–264 on MHC class I [14] (Jackson Labs). *Cbl-b* +/– mice kindly provided by J. Penninger were backcrossed onto the C57BL/6 background for at least ten generations and then crossed with OT-I mice. The resulting *Cbl-b* +/– OT-I mice were then crossed with *Cbl-b* +/– mice to generate *Cbl-b* –/– OT-I mice. To discriminate donor cells and endogenous cells, we bred *Neu*^{OT-I/OT-II} transgenic mice onto a homozygous CD45.1 (Ly5.1) background, whereas donor OT-I T cells expressed CD45.2 (Ly5.2). OT-I mice were also bred with CD90.1 (Thy1.1) congenic mice to allow T cells from these animals to be tracked in CD90.2 (Thy1.2) and CD45.1 host mice. All genotyping was PCR-based [32], and transgenic TCR expression was confirmed by flow cytometry.

Tumor cell lines

The mammary tumor cell lines NOP6, NOP18, NOP21, and NOP23 were derived from spontaneous tumors in transgenic mice expressing *NEU*^{OT-I/OT-II} and a dominant negative version of p53 in mammary epithelium [32] (Martin et al., in preparation). Cell lines were assessed by flow cytometry for expression of H-2 k^b/H-2D^b (Cat. #553575 Pharmingen, Mississauga, ON), I-Ab (Cat. #06261D, Pharmingen), c-NEU (Ab-4, Oncogene Research, San Diego, CA), and SIINFELK/MHC class I (Clone 25-D1.15, a generous gift from Dr. Jonathan Bramson). Tumor cells were injected subcutaneously into the mammary fat pad of 6- to 8-week-old female *Neu*^{OT-I/OT-II} transgenic mice, which lead to the development of 5–70 mm² tumors after 3–6 weeks. Typically, two tumors were implanted in each host animal, contralateral to one another, which provided a further measure of the reproducibility of tumor responses. Note that *Neu*^{OT-I/OT-II} transgenic mice were used as hosts, as WT mice reject NOP tumors due to the immunogenicity of the OT-I and OT-II T cell epitopes [32].

Adoptive transfer and flow cytometry

Single-cell lymphocyte suspensions were prepared from TCR transgenic OT-I donor mice and introduced into host mice via tail vein injection. Unless otherwise indicated, a dose of 15×10^6 splenocytes from OT-I transgenic mice was used for adoptive transfer; this contained approximately 4.5×10^6 CD8⁺ OT-I cells. As NOP21 tumors completely regress after adoptive transfer of this dose of WT OT-I cells, a sub-curative (threefold lower) dose was used to compare the efficacy of WT and CBL-B-deficient OT-I cells in mice bearing NOP21 tumors.

To isolate tumor-infiltrating lymphocytes, tumors were pressed through a 40 μ m membrane, and lymphocytes in the supernatant were stained with fluorescently labeled antibodies to CD8a (Cat. #553034, Pharmingen), CD45.2 (Ly5.2, Cat. #553772, Pharmingen), and CD90.1 (Thy1.1, Cat. #11-0900-85, eBioscience, San Diego, CA). After staining for cell surface markers, intracellular staining was performed for Ki67 expression (Clone SP6, Labvision, Fremont, CA) by fixing cells in fresh 1% paraformaldehyde and permeabilizing with 0.5% Tween 20 in 1% paraformaldehyde. Data were collected using a BD FACSCalibur flow cytometer and analyzed using FlowJo software. Statistical analysis was performed using the Mann Whitney *t* test.

Measurement of tumor responses

Vernier calipers were used to measure tumor size (length \times width). Responses were classified as CR (complete response; no measurable tumor), PR (partial response; >50% tumor reduction), SD (stable disease; <50% reduction or <25% increase), or PD (progressive disease; >25% increase) [32].

Tissue analysis

Tumor tissue was processed following standard methods and either stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry (IHC) with antibodies to NEU (polyclonal antibody, catalogue # 2242, Cell Signaling, Danvers, MA), CD3 (clone SP7, Labvision), Granzyme B (catalogue #ab4059, Abcam, Cambridge, MA) or FOXP3 (clone FJK-16s, eBioscience). Briefly, tissue was fixed in 10% neutral buffered formalin for a minimum of 24 h. The tissue was then processed using a standard overnight protocol on a Thermo Shandon Pathcentre tissue processor, embedded in Paraplast Plus (McCormick Scientific, St Louis, MO), and sectioned at 5 μ m onto Superfrost Plus slides (Fisher Scientific, Ottawa, ON). Following deparaffinization, slides were placed in a Ventana Discovery XT autostainer (Ventana, Tucson, AZ) for IHC. Ventana's standard CC1 protocol was used for antigen retrieval. After incubation with biotinylated

secondary antibody (Jackson ImmunoResearch, West Grove, PA), bound antibodies were detected using the DABMap kit (Ventana) and counterstained with hematoxylin (Ventana). Slides were visualized using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) and images captured using a PAXcam ARC digital microscope camera and PAX-it imaging software Version 6.9 (MIS Inc, Villa Park, IL).

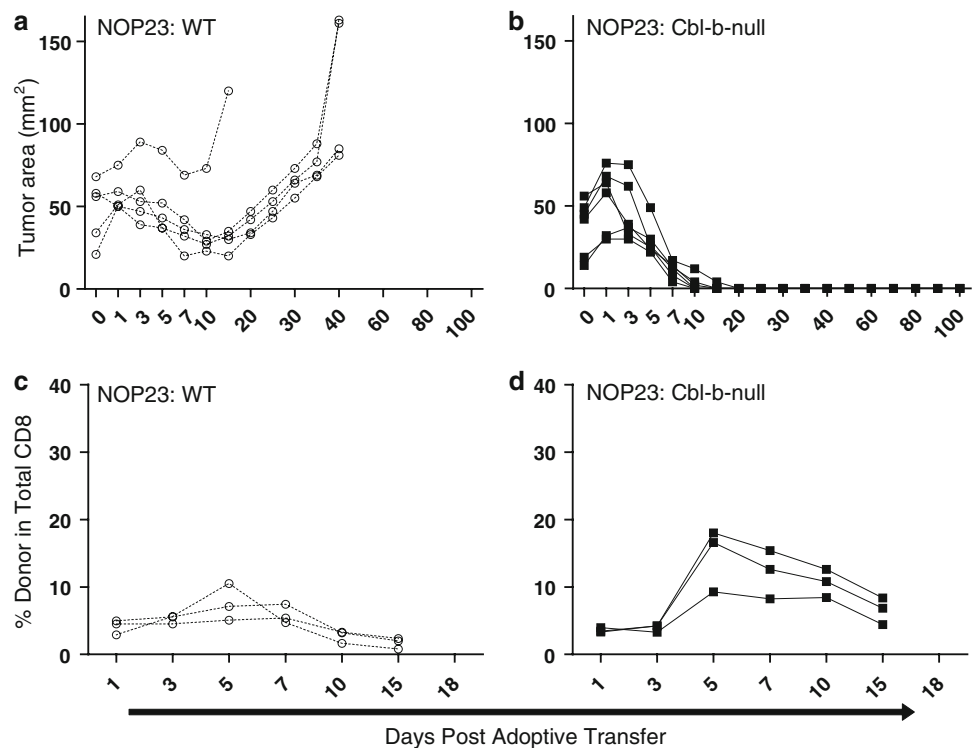
Immune cells were scored using a Chaulkley 49-point grid. Briefly, each immunostained tumor was reviewed under a 40 \times objective with a 49 cross hair grid overlaid on the image. Under a 40 \times objective magnification, this grid defines an area of 0.56 mm². Five epithelial and five stromal fields were evaluated in each case. The proportion of the field occupied by tumor epithelium was estimated using grid cross hairs, as was the total number of positive immune cells within the area of the grid. The number of grid points that coincided with positively staining immune cells within both epithelial and stromal areas was then determined.

Results

A panel of mammary tumor lines with differing sensitivities to CD8⁺ T cells

We have previously shown that female mice co-expressing the *Neu*^{OT-I/OT-II} transgene in mammary epithelium, together with a dominant negative version of p53 (*DNp53*), spontaneously develop mammary adenocarcinomas at 6–10 months of age [32]. We generated a panel of cell lines from such tumors (referred to as “NOP” cell lines, in reference to the components NEU, OVA, and P53). Each cell line was implanted in at least three recipient mice and empirically tested for sensitivity to adoptively transferred OT-I and OT-II cells, as described [32]. While all untreated tumors invariably showed progressive growth, we identified four tumor cell lines with distinct, reproducible responses to adoptively transferred OT-I and OT-II cells. Specifically, NOP21 reproducibly demonstrated a complete response (CR); NOP23 underwent a partial response (PR); and NOP6 and NOP18 demonstrated progressive disease (PD) (Supplementary Fig. 1) [32]. By flow cytometric analysis, all four lines expressed NEU^{OT-I/OT-II}, MHC class I, and the OVA SIINFEKL epitope/MHC class I complex (Supplementary Fig. 2). Thus, the cell lines NOP21, NOP23, NOP6, and NOP18 all express the OT-I and OT-II epitopes, yet differ in the extent of tumor regression after adoptive transfer of OT-I and OT-II cells. This provided a unique model system to evaluate whether *Cbl-b* deficiency can enhance the efficacy of adoptive immunotherapy against phenotypically diverse tumors.

Fig. 1 *Cbl-b*-null CD8⁺ cells demonstrate increased in vivo expansion and enhanced efficacy against a partially responsive tumor. Mice bearing NOP23 mammary tumors underwent adoptive transfer of either WT (a, c) or *Cbl-b*-null (b, d) OT-I cells. Tumor size (a, b) and donor cell number in peripheral blood (c, d) were measured at serial time points after adoptive transfer. For flow cytometry, lymphocytes were gated by forward and side scatter, and CD8⁺ CD45.2⁺ OT-I cells were enumerated relative to total CD8⁺ T cells. X axes are not linear



Cbl-b-null CD8⁺ T cells show enhanced efficacy against a partially responsive tumor

To determine whether *Cbl-b* deficiency could enhance CD8⁺ T cell responses in the NEU^{OT-I/OT-II} mammary tumor model, we generated OT-I cells with homozygous deletion of the *Cbl-b* gene. The anti-tumor activity of *Cbl-b*-null OT-I cells was initially tested against NOP23 tumors, as we reasoned that any enhanced efficacy would be most obvious in the setting of a partial response. Furthermore, adoptive transfers were performed with OT-I cells alone (i.e., without co-transfer of OT-II cells) to create a more challenging experimental setting such that any advantages of *Cbl-b* deficiency would be more pronounced. Mice bearing established NOP23 tumors (≤ 80 mm²) received $\sim 4.5 \times 10^6$ naïve *Cbl-b*-null OT-I cells or, as a control, an equivalent dose of wild type (WT) OT-I cells. As expected, WT OT-I cells induced partial regression of NOP23 tumors within 7–10 days of adoptive transfer, and tumors began to re-grow by day 20 (Fig. 1a). In contrast, *Cbl-b*-null OT-I cells induced complete regression of NOP23 tumors. In 3/3 mice monitored long term, no tumor recurrences were observed for the duration of the experiment (100 days; Fig. 1b). Thus, adoptive transfer of *Cbl-b*-null OT-I cells can induce complete regression of tumors that otherwise demonstrate partial regression.

Cbl-b-null CD8⁺ T cells demonstrate increased expansion in vivo

In time course experiments involving NOP23 tumors, both *Cbl-b*-null and WT OT-I cells underwent clonal expansion between days 3 and 5 (Fig. 1c, d). However, *Cbl-b*-null OT-I cells expanded at a greater rate than WT OT-I cells, as evidenced by the slope of the respective growth curves (5.4 vs. 1.2% per day, respectively; Fig. 1c, d). Consequently, *Cbl-b*-null OT-I cells achieved an approximately twofold higher level in peripheral blood, constituting from 8 to 22% of circulating CD8⁺ cells on day 7 compared to 5–9% for WT OT-I cells (mean 13.5 vs. 7.0, $P = 0.0362$). *Cbl-b*-null OT-I cells also persisted at higher levels than WT OT-I cells (Fig. 1c, d). A subset of mice was sacrificed 7 days after adoptive transfer, and the proliferative status of adoptively transferred cells was determined by flow cytometry for Ki67 expression. When T cells from peripheral blood or lymph node were analyzed, *Cbl-b*-null and WT OT-I cells showed a similar proliferative index (Fig. 2a). Surprisingly, when tumor-infiltrating T cells were analyzed, WT OT-I cells showed a markedly higher proliferative index than *Cbl-b*-null OT-I cells (Fig. 2a), despite being less numerous in peripheral blood.

The above results were potentially confounded by the fact that NOP23 tumors regressed at different rates and to different extents in response to *Cbl-b*-null versus WT OT-I

cells. To make a more direct comparison, we co-transferred *Cbl-b*-null and WT OT-I cells into mice bearing NOP23 tumors. *Cbl-b*-null and WT OT-I cells were distinguished by expression of the Thy-1 congenic marker. As before, *Cbl-b*-null OT-I cells expanded at a greater rate than WT OT-I cells (5.1 vs. 2.5% per day, respectively) and achieved an approximately twofold higher level in peripheral blood (15.3 vs 8.4% of total CD8+ T cells, respectively on day 5, and 13.8 vs. 5.8% on day 7; $P < 0.0001$) (Fig. 2b). When tumor-infiltrating T cells were analyzed, *Cbl-b*-null and WT cells were present in approximately equal numbers on day 5, constituting 55 and 45% of donor CD8+ T cells, respectively (data not shown). By day 7, *Cbl-b*-null OT-I cells were twice as abundant as WT OT-I cells, constituting 66% of donor CD8+ T cells (data not shown). Thus, when compared within the same animal, *Cbl-b*-null OT-I cells achieved approximately twofold higher levels in blood and tumor compared to WT OT-I cells. Despite this difference in abundance, on both days 5 and 7 *Cbl-b*-null and WT OT-I cells showed a nearly identical proliferative index (Ki67) in blood, lymph node, and tumor (Fig. 2c, d). This suggests that the difference in abundance seen between *Cbl-b*-null and WT OT-I cells arises from factors other than proliferation rate, such as different apoptotic rates or trafficking patterns.

Cbl-b-null OT-I cells mediate curative responses against some but not all mammary tumors

We next investigated whether the enhanced expansion and effector activity shown by *Cbl-b*-null OT-I cells against NOP23 tumors applied to mammary tumors with different response characteristics. The NOP21 tumor line normally undergoes complete regression after adoptive transfer of WT OT-I cells. To test whether *Cbl-b*-null OT-I cells show enhanced efficacy against NOP21, mice were given a sub-curative (threefold lower) dose of WT or *Cbl-b*-null OT-I cells. As expected, this dose of WT OT-I cells was insufficient to induce NOP21 tumor regression, and instead tumors grew slowly after adoptive transfer (Fig. 3a). In contrast, the same dose of *Cbl-b*-null cells induced complete, permanent regression of 2/5 tumors, and partial regression of 3/5 tumors (Fig. 3a). This enhanced efficacy was accompanied by a modest increase in T cell expansion, with *Cbl-b*-null OT-I cells reaching 1.3–16.2% of circulating CD8+ cells on day 10, compared to 0.9–1.5% for WT OT-I cells (data not shown).

The NOP6 tumor line normally shows progressive growth after adoptive transfer of WT OT-I cells; mice were therefore given a standard dose ($\sim 4.5 \times 10^6$) of *cbl-b*-null or WT OT-I cells. As expected, WT OT-I cells failed to induce NOP6 tumor regression (Fig. 3b). In contrast, the same dose of *Cbl-b*-null OT-I cells induced complete regression of 3/3 tumors (Fig. 3b). This was accompanied

by increased clonal expansion of OT-I cells in peripheral blood: on day 7, the mean frequency of *Cbl-b*-null OT-I cells was 10.2% of CD8+ cells, while the frequency of WT OT-I cells was 2.5% ($P = 0.0286$; data not shown). In a long-term experiment involving three mice, NOP6 tumors underwent complete regression after treatment with *Cbl-b*-null OT-I cells and did not recur for the duration of the experiment (6 months; data not shown). Thus, *Cbl-b*-null cells can mediate a curative response against a tumor that otherwise demonstrates progressive growth.

Finally, we evaluated the NOP18 tumor line, which also shows progressive growth after adoptive transfer of WT OT-I cells. As expected, after adoptive transfer of a standard dose ($\sim 4.5 \times 10^6$) of WT OT-I cells, 4/4 tumors demonstrated PD (Fig. 3c). In contrast, an equivalent dose of *Cbl-b*-null OT-I cells mediated a modest anti-tumor response (Fig. 3c). Specifically, 1/4 tumors demonstrated SD, and 3/4 tumors demonstrated PD. This was reflected by different tumor growth rates between the WT and *Cbl-b*-null groups (8.0 vs. 2.9 mm²/day, respectively from days 1–15). *Cbl-b*-null OT-I cells also showed increased expansion in peripheral blood: on day 7, the mean frequency of *Cbl-b*-null OT-I cells was 10.8% of CD8+ cells, while the frequency of WT OT-I cells was 3.1% ($P = 0.0286$; data not shown). In a separate experiment, NOP18 tumors failed to regress even after adoptive transfer of a mixture of $\sim 4.5 \times 10^6$ *Cbl-b*-null OT-I cells and $\sim 4.5 \times 10^6$ WT OT-I cells, double the standard dose (data not shown). Thus, *Cbl-b*-null OT-I cells were unable to mediate a curative response against NOP18 tumors.

Tumor infiltration by *Cbl-b*-null OT-I cells

We have previously reported that a significant fraction of NEU^{OT-I/OT-II}-induced tumors are inherently resistant to T cell infiltration and accordingly demonstrate a PD phenotype [32]. To evaluate whether *Cbl-b*-null OT-I cells can overcome such infiltration barriers, we assessed by IHC and Chalkley grid analysis the density of intratumoral CD3+ T cells on day 7 in a subset of mice from the above adoptive transfer experiments. None of the four tumor lines showed significant CD3+ T cell infiltrates prior to adoptive transfer (data not shown). As expected, in mice receiving WT OT-I cells, NOP21 and NOP23 tumors showed the densest T cell infiltration, followed in order by NOP6 and NOP18 tumors (Fig. 4; Table 1). In the case of NOP21, NOP23, and NOP6 tumors, T cells infiltrated both the stromal and epithelial tumor regions (Table 1), and flow cytometry for the CD45 congenic marker revealed that the vast majority were of donor origin (data not shown). In contrast, with NOP18 tumors, infiltrating T cells were few in number, primarily restricted to stroma (Fig. 4; Table 1), and mostly of host origin (i.e., 60% host vs. 40% donor; data not shown).

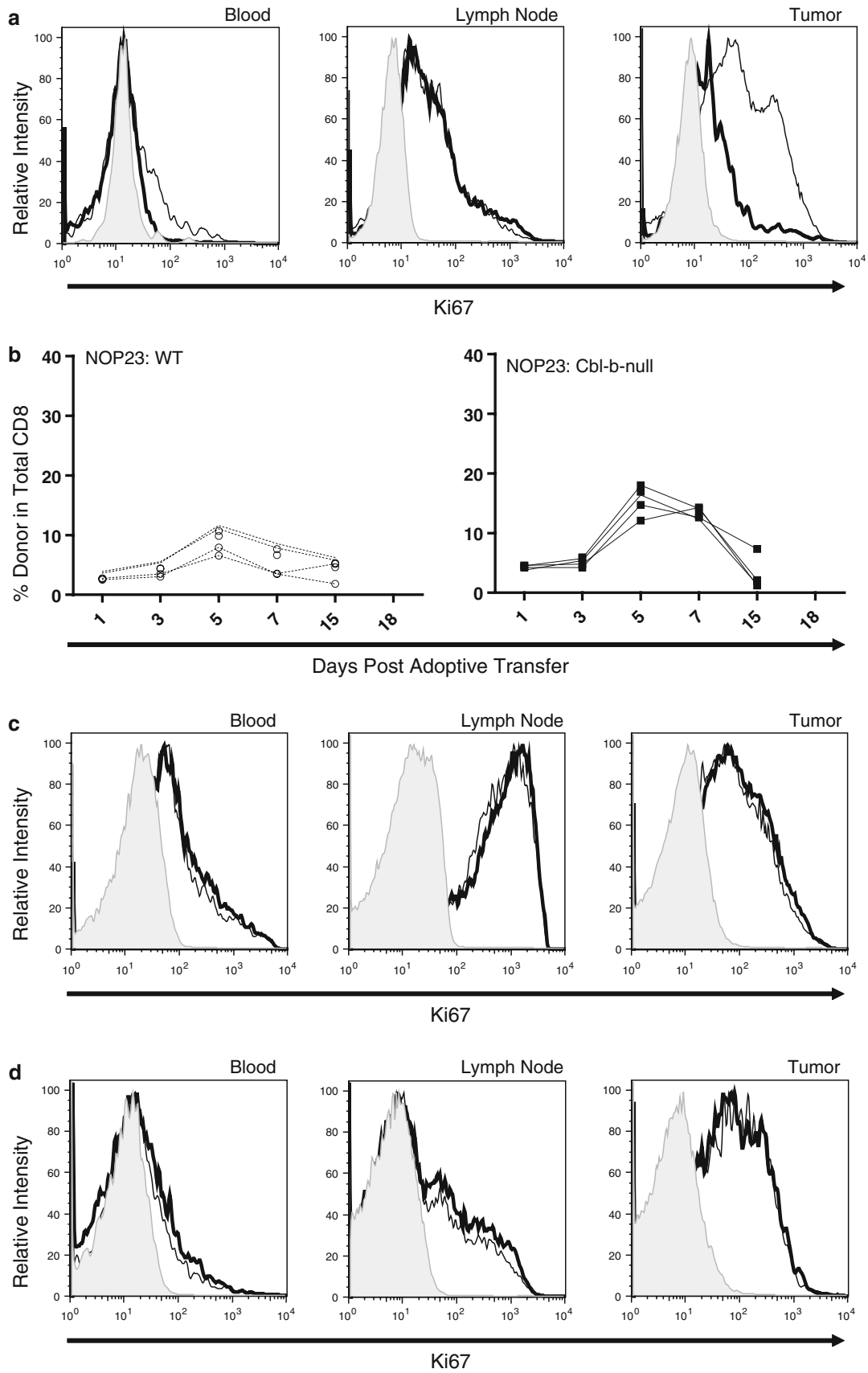


Fig. 2 Proliferation of adoptively transferred WT and *Cbl-b*-null OT-I cells in mice bearing NOP23 mammary tumors. **a** Expression of Ki67 by adoptively transferred OT-I cells in different tissue compartments. *Cbl-b*-null and WT OT-I cells were transferred into separate host mice, and tissues were harvested 7 days later. Lymphocytes were gated by forward and side scatter, and analyzed for expression of CD8, CD45.2 (which marks all donor cells), and Ki67, a marker of proliferation, compared to cells stained with secondary antibody alone (*shaded*). The proliferative index of WT (*thin line*) and *Cbl-b*-null (*heavy line*) OT-I cells was similar in blood and lymph node, whereas tumor-infiltrating WT OT-I cells had a higher proliferative index. **b-d** Proliferation of *Cbl-b*-null and WT OT-I cells co-transferred into the same host mice. Lymphocytes were gated by forward and side scatter, and analyzed for expression of CD8a, CD45.2 (which marks all donor cells), and CD90.1 (which distinguishes WT from *Cbl-b*-null OT-I cells). **b** Expansion of OT-I cells in peripheral blood relative to total CD8+ T cells. **c, d** Expression of Ki67 by WT and *Cbl-b*-null OT-I cells in different tissue compartments on day 5 (**c**) and day 7 (**d**). Control cells were stained with secondary antibody alone (*shaded*). X-axes are not linear

Compared to WT OT-I cells, adoptive transfer of *Cbl-b*-null OT-I cells resulted in enhanced T cell infiltration of NOP21 and NOP6 tumor epithelium (i.e., 1.5-fold

increase for NOP21, and 2.8-fold increase for NOP6), whereas infiltration of tumor stroma was unchanged for both NOP21 and NOP6 (Table 1). For NOP23 tumors, there was actually a 20% decrease in infiltration of tumor epithelium with *Cbl-b*-null OT-I cells compared to WT OT-I cells, probably because the tumors had largely regressed by day 7 in the former case (Table 1). For NOP18 tumors, *Cbl-b*-null OT-I cells failed to enhance the overall infiltration of tumor epithelium or stroma (Table 1), and the relative proportion of host and donor T cells was essentially unchanged. Thus, *Cbl-b*-null OT-I cells demonstrated enhanced infiltration of some but not all mammary tumors.

Finally, tissue sections from the above studies were stained with antibodies to GRANZYME B and FOXP3, which are markers of cytolytic and regulatory T cells, respectively. In general, tumors contained very few GRANZYME B+ cells, and there was no obvious difference between the WT and *Cbl-b*-null groups (data not shown). In contrast, FOXP3+ cells were sufficiently numerous to be quantified by Chaukley grid analysis,

Fig. 3 *Cbl-b*-null CD8+ cells demonstrate enhanced efficacy against some but not all mammary tumors. Mice bearing NOP21 (**a**), NOP6 (**b**), or NOP18 (**c**) mammary tumors underwent adoptive transfer of either WT (*left panels*) or *Cbl-b*-null (*right panels*) OT-I cells. Tumor size was measured at serial time points after adoptive transfer. Mice bearing tumors demonstrating progressive growth despite adoptive transfer of OT-I cells were sacrificed 2–4 weeks post-adoptive transfer, depending on tumor growth rates. X axes are not linear

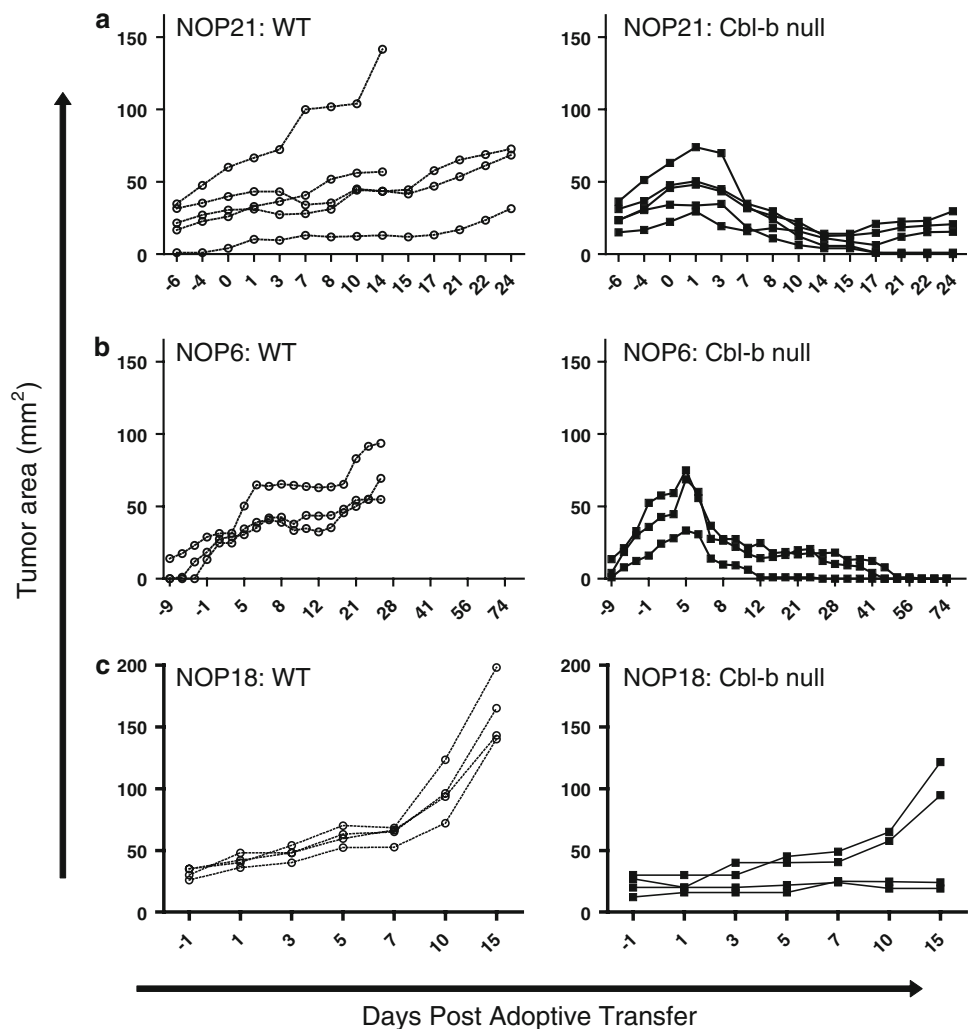
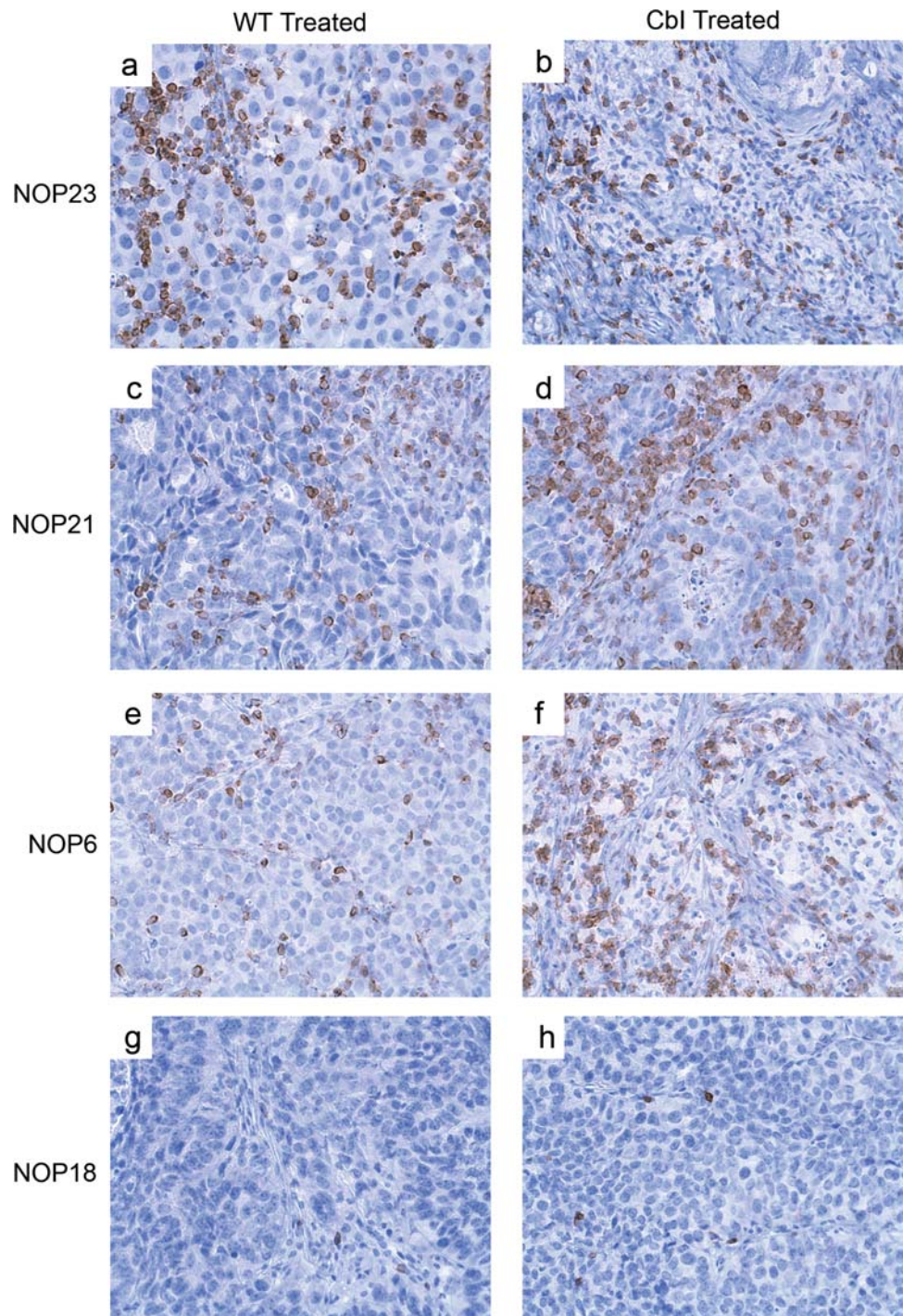


Fig. 4 *Cbl-b*-null CD8⁺ cells demonstrate enhanced infiltration of some but not all mammary tumors. Immunohistochemical analysis of CD3⁺ T cells 7 days after adoptive transfer of WT (*left panels*) or *Cbl-b*-null (*right panels*) OT-I cells into mice bearing **a, b** NOP23 tumors, **c, d** NOP21 tumors, **e, f** NOP6 tumors, or **g, h** NOP18 tumors



which allowed their abundance relative to CD3⁺ T cells to be estimated. For tumors treated with WT OT-I cells, FOXP3⁺ cells represented anywhere from 4.2 to 24.3% of CD3⁺ cells in the tumor epithelium depending on the tumor cell line (Table 1). In contrast, after adoptive transfer of *Cbl-b*-null OT-I cells, FOXP3⁺ cells were proportionately less numerous in all tumors except NOP18, although these trends did not reach statistical

significance possibly due to low sample size (Table 1). The effect was most striking for NOP6, in which 24.3% of intraepithelial CD3⁺ cells were FoxP3⁺ after adoptive transfer of WT OT-I cells, versus 8.4% with *Cbl-b*-null OT-I cells (Table 1). Thus, adoptive transfer of *Cbl-b*-null OT-I cells may trigger proportionately less infiltration of the tumor epithelium by FoxP3⁺ cells in some tumors.

Table 1 Frequency of CD3+ and FOXP3+ tumor infiltrating T cells in mice after adoptive transfer of WT or *Cbl-b*-null cells

	NOP21	<i>P</i> value	NOP23	<i>P</i> value	NOP6	<i>P</i> value	NOP18	<i>P</i> value
Intraepithelial								
CD3+ cells								
WT	46.5	ns	82.7	ns	27.4	0.0286	3.1	ns
<i>Cbl-b</i> -null	67.8		65.7		75.6		3.5	
FOXP3+ cells								
WT	4.9	ns	6.2	0.0294	6.5	ns	0.1	ns
<i>Cbl-b</i> -null	5.5		2.2		6.2		0.1	
%FOXP3/CD3								
WT	10.9	ns	7.4	ns	24.3	ns	4.2	ns
<i>Cbl-b</i> -null	8.4		3.3		8.4		3.6	
Intrastromal								
CD3+ cells								
WT	64.3	ns	72.1	ns	52.8	ns	30.0	ns
<i>Cbl-b</i> -null	69.3		56.5		53.8		33.9	
FOXP3+ cells								
WT	9.4	ns	1.5	ns	11.5	0.0286	4.6	ns
<i>Cbl-b</i> -null	12.6		1.7		3.4		5.3	
%FOXP3/CD3								
WT	14.7	ns	2.1	ns	22.0	0.0286	16.0	ns
<i>Cbl-b</i> -null	18.1		2.6		6.2		15.5	

ns not significant

Discussion

We have evaluated the efficacy of *Cbl-b*-null CD8+ T cells against a panel of phenotypically diverse mammary tumors. In general, *Cbl-b*-null OT-I cells showed enhanced expansion and persistence in peripheral blood compared to WT OT-I cells (Fig. 1). For three of four tumor lines, this was accompanied by enhanced infiltration of tumor epithelium (Fig. 4; Table 1) and an improved anti-tumor response (Figs. 1, 3). The most striking results were seen with NOP6 tumors, which showed progressive growth in the face of WT OT-I cells yet completely regressed when challenged with *Cbl-b*-null OT-I cells. By contrast, *Cbl-b*-null OT-I cells were unable to eliminate a second PD tumor line (NOP18) due to a profound infiltration barrier that restricted T cells to tumor stroma (Fig. 4; Table 1). Thus, not all tumor phenotypes are permissive to infiltration and anti-tumor activity by *Cbl-b*-null T cells.

In time course experiments with NOP23 tumors, *Cbl-b*-null OT-I cells expanded to an approximately two-fold higher level in peripheral blood compared to WT OT-I cells (Figs. 1c, d, 2b). Similarly, *Cbl-b*-null CD8+ T cells have shown modestly enhanced primary expansion in other models, including peptide immunization [15] and LCMV infection [12], whereas primary expansion was similar to WT CD8+ T cells in two other LCMV studies [25, 30]. In another study, when CD4+ *Cbl-b*-null T cells were stimulated by peptide in vivo, they proliferated at about the same rate as WT cells, but continued proliferating for an extra

day, resulting in approximately one extra cell division [40]. Thus, *Cbl-b*-null T cells appear to have only a modest proliferative advantage over WT T cells in vivo. In the present study, despite undergoing a two-fold difference in overall expansion, co-transferred *Cbl-b*-null and WT OT-I cells showed a nearly identical proliferative index (as measured by Ki67 staining) in blood, lymph node, and tumor on days 5 and 7 (Fig. 2c, d). There are several possible explanations for this apparent discrepancy. First, a two-fold difference in expansion reflects a difference of only one cell division between *Cbl-b*-null and WT OT-I cells, which may be difficult to detect by Ki67 staining. Second, *Cbl-b*-null OT-I cells may have had a higher proliferative index at a time point earlier than day 5. Third, WT and *Cbl-b*-null OT-I cells may have a similar proliferative rate but different apoptotic rates. While a formal possibility, it is noteworthy that studies in an LCMV model showed similar Annexin V expression by WT and *Cbl-b*-null CD8+ T cells [30]. Finally, WT OT-I cells might have preferentially trafficked to tissue compartments other than those analyzed (lymph node, blood, and tumor), therefore seeming to be less abundant than *Cbl-b*-null OT-I cells. Irrespective of the underlying mechanism, the modest difference in cell number between WT and *Cbl-b*-null T cells suggests that other mechanisms likely contribute to the enhanced efficacy of *Cbl-b*-null OT-I cells.

Compared to WT OT-I cells, *Cbl-b*-null cells displayed dramatically enhanced infiltration of the epithelial regions of NOP21 and NOP6 tumors (Fig. 4; Table 1). We suspect

that *Cbl-b*-null OT-I cells also infiltrated NOP23 tumors more extensively, given the striking tumor regression that occurred by day 7. Improved tumor infiltration by adoptively transferred *Cbl-b*-null cells has also been observed in E.G7 lymphomas [4], and might be attributable to the increased adhesion of *Cbl-b*-null T cells to ICAM-1 [41]. Similarly, *Cbl-b*-null macrophages show increased activation of VAV1 and LFA-1, which results in enhanced adhesion to endothelial cells and greater tissue infiltration in models of diabetes and peritonitis [6, 13, 35]. Importantly, however, CBL-B deficiency is not sufficient to overcome all infiltration barriers, as OT-I cells remained restricted to stromal regions in NOP18 tumors, unable to penetrate the malignant epithelium. We are currently investigating the infiltration barrier that distinguishes NOP18 tumors and leads to this exceptionally immune-resistant phenotype.

Adoptive transfer of either *Cbl-b*-null or WT OT-I cells induced marked infiltration of tumors by FOXP3+ cells (Table 1). We analyzed a subset of tumors and found that FOXP3+ cells were exclusively of host origin, as manifested by expression of host congenic markers (data not shown). In the case of NOP23 and NOP6 tumors, the FOXP3+ cell infiltrate was less dense after adoptive transfer of *Cbl-b*-null OT-I cells compared to WT OT-I cells, suggesting a more favorable effector:Treg ratio in the former case. In NOP23 tumors, this difference appeared to arise from a reduced absolute number of FOXP3+ cells in the tumor bed of animals treated with *Cbl-b*-null OT-I cells (Table 1). Recruitment of FOXP3+ cells to sites of infection, inflammation, or malignancy is mediated largely by chemokines [34]. Furthermore, conventional CD4+ T cells can be converted to a FOXP3+ regulatory phenotype by factors such as TGF- β [3]. One can speculate that such factors may be less abundant in the inflammatory milieu created by *Cbl-b*-null OT-I cells compared to WT cells. One can further speculate that the reduced proportion of FOXP3+ cells could contribute to the increased efficacy of *Cbl-b*-null OT-I cells. This effect may be further enhanced by the intrinsic resistance of *Cbl-b*-null T cells to suppression by FOXP3+ T cells [21, 36] and the immunosuppressive cytokine TGF- β [4, 21, 36, 37].

A major implication of this study is that *Cbl-b*-null T cells, while showing enhanced efficacy against some tumors, are unable to mediate curative responses against all tumor phenotypes, especially those with strong infiltration barriers as exemplified by NOP18. When considering clinical translation of this strategy, one must consider whether the potential advantages of using *Cbl-b*-null T cells outweigh the technical challenges and potential risks associated with the genetic modification of T cells. Our results show that the enhanced efficacy of *Cbl-b*-null T cells is associated with relatively modest increases in cell proliferation and tumor infiltration and decreased regulatory T cell

recruitment. Equivalent results could potentially be achieved by combining adoptive immunotherapy with standard treatments. For example, in human breast and ovarian cancer, taxane-based chemotherapy can enhance tumor infiltration by CD3+ and CD8+ T cells to a similar or greater extent as seen in the present study [7, 18, 22]. We are currently evaluating whether chemotherapy can enhance the anti-tumor activity of OT-I cells to a similar extent as seen with *Cbl-b* deficiency, thereby providing a clinically feasible alternative to T cell engineering.

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