Spontaneous tumor rejection by *cbl-b*–deficient CD8$^+$ T cells

Stefanie Loeser,[1] Karin Loser,[2] Martijn S. Bijker,[3] Manu Rangachari,[1] Sjoerd H. van der Burg,[4] Teiji Wada,[1] Stefan Beissert,[2] Cornelis J.M. Melief,[3] and Josef M. Penninger[1]

[1]Institute of Molecular Biotechnology of the Austrian Academy of Science, 1030 Vienna, Austria
[2]Department of Dermatology and Interdisciplinary Center of Clinical Research, University of Münster, D-48149 Münster, Germany
[3]Department of Immunohematology and Blood Transfusion and [4]Department of Clinical Oncology, Leiden University Medical Centre, 2333 ZA Leiden, Netherlands

The concept of tumor surveillance implies that specific and nonspecific components of the immune system eliminate tumors in the early phase of malignancy. Understanding the biochemical mechanisms of tumor immunosurveillance is of paramount significance because it might allow one to specifically modulate spontaneous antitumor activity. We report that inactivation of the E3 ligase Casitas B cell lymphoma-b (Cbl-b) confers spontaneous in vivo rejection of tumor cells that express human papilloma virus antigens. Moreover, *cbl-b*$^{-/-}$ mice develop significantly fewer ultraviolet B (UVB)–induced skin malignancies and reject UVB-induced skin tumors. CD8$^+$ T cells were identified as key players in the spontaneous tumor rejection response. Loss of Cbl-b not only enhances antitumor reactivity of CD8$^+$ T cells but also occurs in the absence of CD4$^+$ T cells. Mechanistically, *cbl-b*$^{-/-}$ CD8$^+$ T cells are resistant to T regulatory cell–mediated suppression and exhibit enhanced activation and rapid tumor infiltration. Importantly, therapeutic transfer of naive *cbl-b*$^{-/-}$ CD8$^+$ T cells is sufficient to mediate rejection of established tumors. Even up to 1 yr after the first encounter with the tumor cells, *cbl-b*$^{-/-}$ mice carry an "anticancer memory." These data identify Cbl-b as a key signaling molecule that controls spontaneous antitumor activity of cytotoxic T cells in different cancer models. Inhibition of Cbl-b is a novel approach to stimulate long-lasting immunity against cancer.

CORRESPONDENCE
Josef M. Penninger:
Josef.penninger@imba.oeaw.ac.at

Abbreviations used: Cbl-b, Casitas B cell lymphoma-b protein; HPV, human papilloma virus.

More than 100 yr ago, it was discovered that tumors regress in patients injected with bacterial extracts (1), suggesting that immune cells might be capable of eliminating cancer cells in the early phase of malignancy (2, 3). In many cases, tumor growth and lack of anticancer immunity can be ascribed to the fact that tumor cells do not provide sufficient T cell stimulation or induce tolerance in the tumor-reactive T cell population (4–7). Several attempts have been made to break such tumor tolerance and to specifically enhance antitumor immunity by modulating immune cells (8, 9). However, immunotherapy is still difficult because most therapies result in severe side-effects, require large amounts of immune cells, or depend on extensive genetic manipulations of effector cell populations. Thus, identification of a key dominant "tolerogenic" factor in T cells that directly controls activation of tumor-reactive cytotoxic T cells in vivo might circumvent these limitations of T cell immunotherapy.

The Casitas B cell lymphoma-b (Cbl-b) protein is a member of the mammalian family of Cbl E3 ubiquitin ligases (10). Proteins of this family contain an N-terminal tyrosine kinase binding domain, a RING finger, and a C-terminal proline-rich sequence, and can thus function as both E3 ligases and molecular adaptors (10). Studies of Cbl-b–deficient mice have revealed an essential role for this molecule in T-cell tolerance induction. *Cbl-b*$^{-/-}$ T cells show effective activation in the absence of costimulation, resulting in spontaneous autoimmunity or enhanced susceptibility to autoantigens (11–14). Moreover, Cbl-b sets the threshold for T cell activation to "weak" antigens (11, 15, 16) and controls immunotolerance in multiple experimental systems in vitro and in vivo (13, 14, 17, 18).

K. Loser and M.S. Bijker contributed equally to this work.
The online version of this article contains supplemental material.

Thus, Cbl-b functions as a negative regulator of antigen-specific T cell activation and is a critical mediator of T cell anergy. Based on these findings, we hypothesized that Cbl-b–regulated T cell activation may hold the key to our understanding of induction and/or maintenance of T cell responses to cancer cells.

RESULTS

Cbl-b mutant mice spontaneously reject tumors

To determine whether Cbl-b contributes to anticancer immunity in vivo, we tested the TC-1 cancer model in *cbl-b*–deficient mice. TC-1 cells are c-H-ras–transformed, C57BL/6-syngeneic fibroblasts expressing the human papilloma virus (HPV) 16–derived oncoproteins E6 and E7 as tumor-relevant T cell antigens (19). High-risk HPV infection is a major cause of cervical cancer in women, with a high mortality rate, and the HPV-16 E6 and E7 oncoproteins are almost invariably expressed in early cervical cancer (20). Importantly, HPV vaccinations can protect against cervical cancer, indicating that immunoreactivity against HPV antigens plays a key role in cancer prevention and therapy (21).

In mice, injection of HPV-16 E6- and E7-expressing TC-1 tumor cells into syngeneic recipients results in rapid tumor growth that can be abrogated by vaccination with an E7-peptide–based vaccine (22, 23). Therefore, we injected TC-1 subcutaneously into the left flank of wild-type (*cbl-b*$^{+/+}$), *cbl-b* heterozygous (*cbl-b*$^{+/-}$), and *cbl-b*–deficient (*cbl-b*$^{-/-}$) mice. In all recipients, tumor growth was first macroscopically observed at ~3–5 d after inoculation of 2.5×10^5 tumor cells (Fig. 1 A). As previously reported, in naive C57BL/6 mice (24), the tumors continued to grow progressively and with similar kinetics in all *cbl-b*$^{+/+}$ and *cbl-b*$^{+/-}$ mice analyzed (Fig. 1, A and B). The histology of tumors, kinetics of tumor growth, and tumor incidences were comparable between *cbl-b*$^{-/-}$, *cbl-b*$^{+/-}$, and *cbl-b*$^{+/+}$ mice in the first 2 wk after tumor inoculation (Fig. 1 C and not depicted). Intriguingly, starting at ~2 wk after TC-1 inoculation, naive *cbl-b*$^{-/-}$ mice spontaneously rejected the tumors (Fig. 1 A). Tumor mass progressively reduced in *cbl-b*$^{-/-}$ mice and became undetectable between 25 and 35 d after the initial inoculation of tumor cells (Fig. 1 C). At 3 wk after inoculation, the average size of a wild-type tumor was 835 mm^3 (± 227.9 mm^3 SEM; $n = 5$) compared with an average size of 14 mm^3 (± 7.6 mm^3 SEM; $n = 5$; $P < 0.003$) in *cbl-b*$^{-/-}$ recipients (Fig. 1 D). Over 80% of *cbl-b*$^{-/-}$ mice completely rejected the tumors and remained tumor free throughout the experimental observation period that, in some cases, was longer than 1 yr (Fig. 1 B and not depicted). Progressive tumor growth in *cbl-b*$^{-/-}$ mice was observed in a few cases, but only after a longer latency period compared with tumor growth in wild-type mice (Fig. 1, A and B). It should be noted that we injected a tumor cell number (2.5×10^5) into our experimental cohorts that is 10 times higher than the dose that is lethal for wild-type mice (24). These surprising data show that naive *cbl-b* mutant mice can spontaneously reject a very high dose of aggressive TC-1 tumor cells.

Spontaneous tumor rejection in *cbl-b* mutant mice is mediated by CD8$^+$ T cells

To explore the underlying mechanisms of spontaneous tumor rejection in the *cbl-b* knockout mice, we assessed proliferation and cell death of tumor tissue. Tumor cell proliferation was comparable in both *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice on days 7, 14 (Fig. 2 A), and 21 (not depicted), suggesting that loss of Cbl-b expression in the host environment does not affect cell cycle progression of the tumor cells. In contrast, whereas cell death within early tumors (7 d after inoculation) appeared comparable among the different cohorts, we observed markedly increased apoptosis in day 14 tumors taken from *cbl-b*$^{-/-}$ mice (Fig. 2 B). We next determined the numbers of lymphoid cells in tumor-bearing *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice. We did not observe alterations in CD11b$^+$, CD11c$^+$, NK1.1$^+$ cells, or Gr1$^+$ granulocytes, nor in relative numbers of CD4$^+$ or CD8$^+$ T cells in the draining inguinal lymph nodes, nondraining contralateral inguinal lymph nodes, or the spleen (not depicted). Immunohistochemistry (not depicted) and FACS analysis (Fig. 2 C) of total tumor tissue revealed that CD4$^+$ and CD8$^+$ T cells infiltrate the tumors in both wild-type and *cbl-b*$^{-/-}$ mice. However, we observed markedly increased ratios of CD8$^+$ within the tumors of *cbl-b*$^{-/-}$ mice compared with wild-type mice (Fig. 2 C). Of note, we observed tumor infiltration of CD8$^+$ cells as early as 8 d after TC-1 inoculation in *cbl-b*$^{-/-}$ mice (Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20061699/DC1). In line with increased CD8$^+$ T cell infiltration of tumors, we also detected elevated levels of the CD8$^+$ T-cell chemokine RANTES (25) in tumors growing in *cbl-b*$^{-/-}$ mice (not depicted). These data show that loss of Cbl-b expression in mice results in increased tumor cell death, increased infiltration of CD8$^+$ T cells into the tumor tissue, and, most importantly, spontaneous tumor rejection.

To investigate whether CD8$^+$ T cells from wild-type and *cbl-b*$^{-/-}$ mice were reactive to the tumor-specific antigens, we analyzed IFNγ production by CD8$^+$ T cells isolated from the spleen (not depicted) and lymph nodes (Fig. 3 A) of tumor-bearing mice upon restimulation with the MHC class I ($H2D^b$) restricted E7 tumor-specific peptide antigen. In the draining lymph nodes of TC-1 challenged wild-type mice, we consistently observed a low frequency of E7-reactive, CD8$^+$, IFNγ-producing T cells. Importantly, in all tumor-bearing *cbl-b*$^{-/-}$ mice analyzed, the frequency of IFNγ-producing CD8$^+$ T cells was markedly increased in response to stimulation with the E7 peptide (Fig. 3 A). Of note, we also observed E7 tumor-specific, IFNγ-producing CD8$^+$ T cells, albeit at lower numbers, in the nondraining contralateral inguinal lymph nodes of *cbl-b*$^{-/-}$ mice. These data show that spontaneous tumor rejection in *cbl-b* mutant mice is associated with rapid CD8$^+$ T cell infiltration into the tumor and hyperactivation of tumor-specific cytotoxic T cells.

To examine whether CD8$^+$ T cells are, indeed, essential for the spontaneous rejection of TC-1 tumor cells in *cbl-b* mutant mice, we depleted CD8$^+$ cells using specific antibodies before the tumor injection. After confirmation of CD8$^+$ T cell depletion

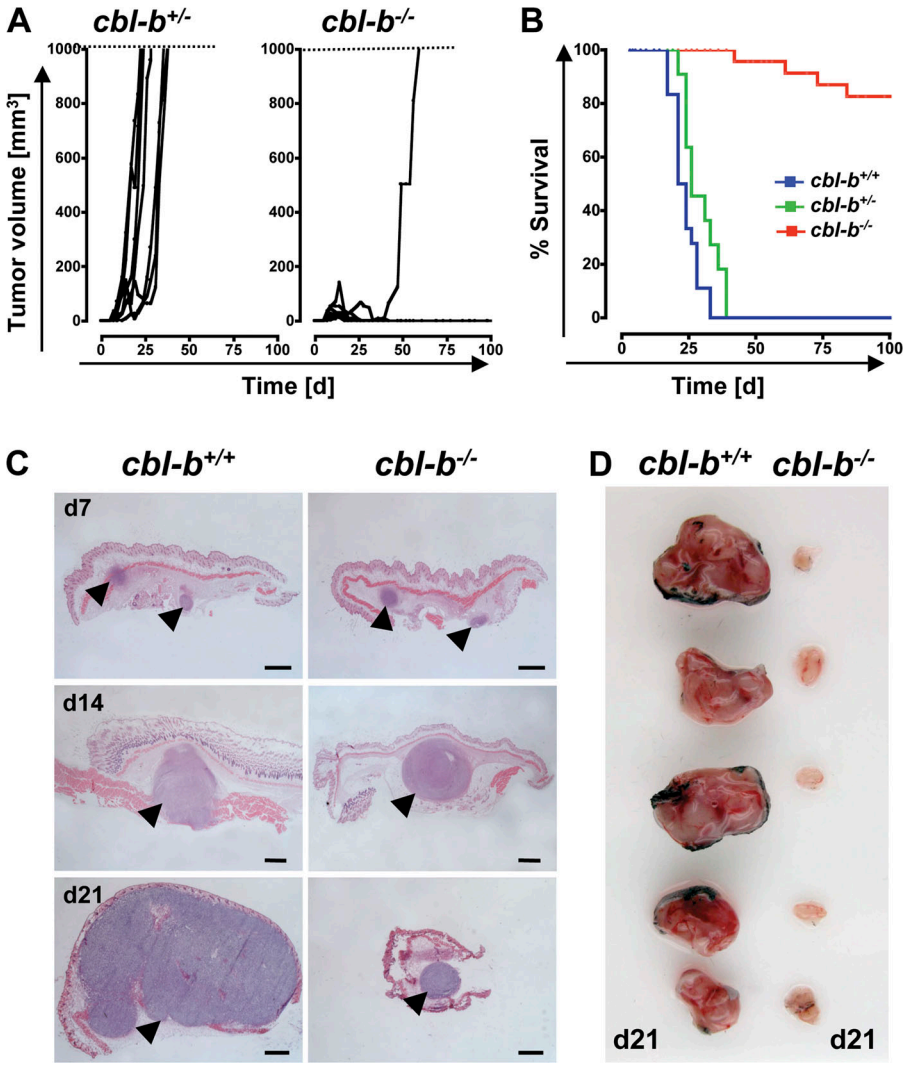

Figure 1. Spontaneous tumor rejection in *cbl-b*$^{-/-}$ mice. (A) Kinetics of TC-1 tumor cell growth in *cbl-b*$^{+/-}$ ($n = 6$) and *cbl-b*$^{-/-}$ ($n = 7$) mice. 2.5×10^5 TC-1 cells were injected into the flanks of 8–12-wk-old littermate mice, and tumor volume was measured with a caliper (mm^3) over time (days). Of note, only mice that developed a palpable tumor were included in the experimental cohorts. (B) Kaplan-Meier survival curves of *cbl-b*$^{+/+}$ ($n = 18$), *cbl-b*$^{+/-}$ ($n = 11$), and *cbl-b*$^{-/-}$ ($n = 28$) mice inoculated with 2.5×10^5 TC-1 tumor cells. Data are pooled from four different experiments. (C) Representative histology of TC-1 tumors isolated on different days (7, 14, and 21 d after inoculation) from *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice. Hematoxylin and eosin staining. Arrows point at tumor mass. Bars, 1 mm. (D) Macroscopic appearance of TC-1 tumors in 5 different *cbl-b*$^{+/+}$ and 5 different *cbl-b*$^{-/-}$ mice 21 d (d21) after inoculation.

(Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061699/DC1), *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice were injected with TC-1 tumors and tumor growth was monitored for up to 1 yr. Tumors grew progressively in the *cbl-b*$^{+/+}$ control mice, and the kinetics of tumor expansion in the CD8$^+$ T-cell–depleted group was comparable to the control cohort (Fig. 3 B). In the *cbl-b*$^{-/-}$ mice, tumors were spontaneously rejected in the control group, with some cases of late-onset of tumor growth (Fig. 3 B; and Fig. 1, A and B). Importantly, CD8$^+$ T cell–depleted *cbl-b*$^{-/-}$ mice displayed progressive and lethal tumor growth (Fig. 3 B).

In most experimental models of tumor rejection, CD4$^+$ T cell help is required for effective antitumor immunity (26, 27). Similarly, it has been shown that the vaccination-induced anti–TC-1 tumor response depends on CD4$^+$ T cell help (22). To address the role of CD4$^+$ T cells in spontaneous tumor rejection, we efficiently depleted CD4$^+$ cells in *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice (Fig. S2), followed by TC-1 inoculation. As expected, depletion of CD4$^+$ T cells in wild-type mice did not change the kinetics or frequencies of tumor growth. Surprisingly, ablation of CD4$^+$ T cells in *cbl-b*$^{-/-}$ mice did not affect their capacity to spontaneously reject the tumor (Fig. 3 B), indicating that CD4$^+$ T cells are not required for rejection of TC-1 tumors in our experimental system. These data show that CD8$^+$ T cells play an essential role in the spontaneous rejection of TC-1 tumors in *cbl-b* mutant animals.

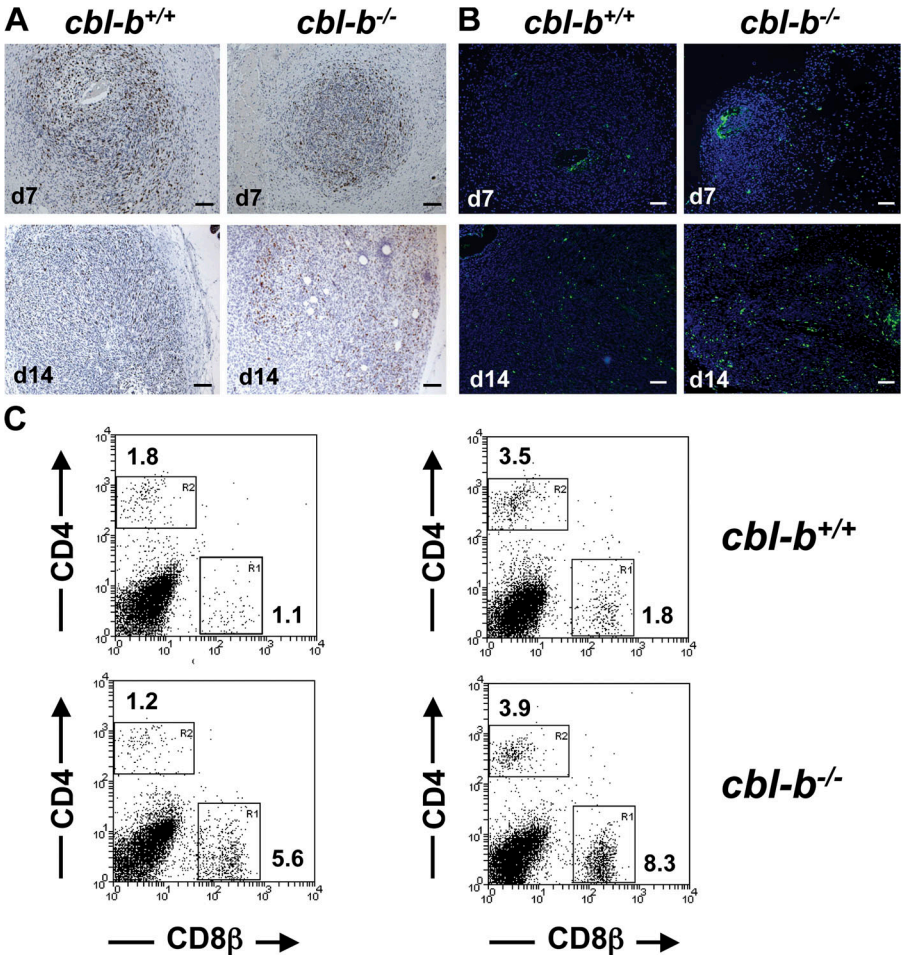

Figure 2. Infiltration of CD8$^+$ T cells into tumors from *cbl-b*$^{-/-}$ mice. (A) Immunohistochemistry for proliferation marker Ki67 in TC-1 tumor samples from *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice 7 and 14 d after tumor inoculation (2.5×10^5). (B) Increased cell death in tumor tissue from *cbl-b*$^{-/-}$ mice 14 d after TC-1 tumor cell injection. Cell death was determined by TUNEL. Representative images of individual mice on day 7 and 14 are shown. (C) Analysis of tumor-infiltrating lymphocytes 17 (left) and 21 d (right) after TC-1 inoculation into *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice. Single-cell suspensions were analyzed by flow cytometry using antibodies reactive to CD8β and CD4. Numbers indicate percentages of cells within the R1 and R2 gates. Bars, 100 μm.

Therapeutic transfer of naive *cbl-b*$^{-/-}$ CD8$^+$ T cells is sufficient to mediate spontaneous rejection of established tumors

Our data show that *cbl-b* mutant mice spontaneously reject TC-1 tumors via CD8$^+$ T cells. We then wanted to ask whether *cbl-b*$^{-/-}$ CD8$^+$ cells could also be used to treat a previously established cancer. To address whether *cbl-b*$^{-/-}$ CD8$^+$ cells function therapeutically, we set up an adoptive transfer model. In this model, T- and B-cell–deficient *rag2* mutant mice were injected with 2.5×10^5 TC-1 tumor cells, followed by infusion of 3×10^6 and 2×10^6 purified CD8$^+$ T cells from naive *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ donors on days 3 and 6, respectively, after initial tumor cell challenge. In this experimental system, tumors grow progressively in a wild-type environment, followed by two therapeutic vaccinations with freshly purified, polyclonal, and naive CD8$^+$ T cells from syngeneic donors. Tumor growth was monitored for a period of 6 wk (Fig. 4). The kinetics and extent of TC-1 tumor growth was comparable between *rag2*$^{-/-}$ control mice and *rag2*$^{-/-}$ mice infused with wild-type CD8$^+$ T cells (Fig. 4 A). In contrast, tumor growth was markedly reduced and delayed in *rag2*$^{-/-}$ mice that received *cbl-b*$^{-/-}$ CD8$^+$ T cells as a therapeutic vaccine (Fig. 4 A). Moreover, although the same numbers of cells were transferred from wild-type and *cbl-b*$^{-/-}$ donors, we observed a marked increase in the numbers of CD8$^+$ *cbl-b*$^{-/-}$ T cells in the blood of *rag2*$^{-/-}$ hosts (Fig. 4 B). These data show that therapeutic transfer of naive *cbl-b*$^{-/-}$ CD8$^+$ T cells is sufficient to mediate spontaneous rejection of established tumors.

Tumor escape in *cbl-b*$^{-/-}$ mice

Recent results suggested that the dominant mechanism of spontaneous tumor growth is induction of immunotolerance rather than immunoescape of the tumor cells (28). However, Cbl-b appears to be a critical regulator of antigen-specific T cell tolerance, and it has been shown in multiple systems

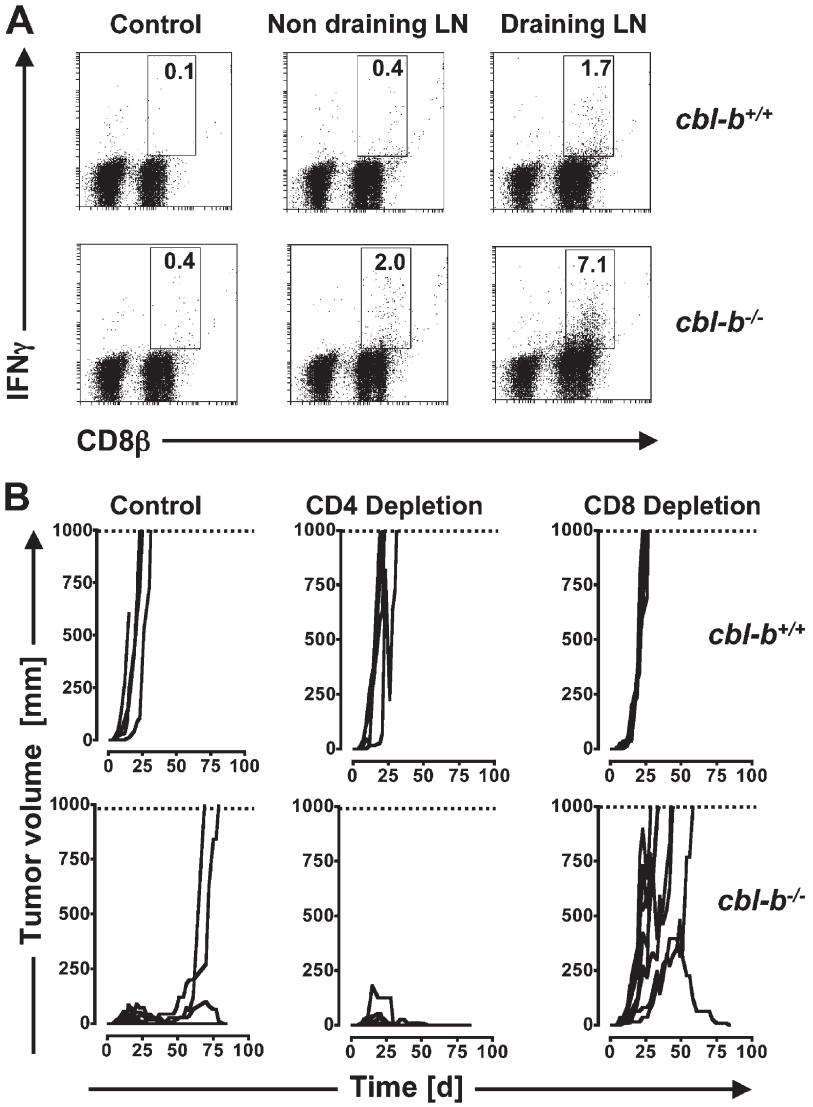

Figure 3. CD8$^+$ cells mediate spontaneous tumor rejection independent of CD4$^+$ T cell help. (A) INFγ production of CD8$^+$ T cells isolated from draining and nondraining inguinal lymph nodes of tumor-bearing *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice ($n = 5$) and naive control mice ($n = 3$). Data are from 21 d after tumor inoculation. Purified CD8β$^+$ T cells were restimulated for 60 h with the HPV-16–derived peptide E7$^{49-57}$, stained for intracellular IFNγ, and analyzed by flow cytometry. Numbers indicate percentages of INFγ$^+$ CD8β$^+$ T cells. (B) Kinetics of TC-1 tumor cell growth in *cbl-b*$^{+/+}$ (top; $n = 6$) and *cbl-b*$^{-/-}$ (bottom; $n = 7$) mice left untreated or after depletion of CD4$^+$ or CD8$^+$ T cell subsets. 2.5×10^5 TC-1 cells were injected into the flanks of 8–12-wk-old mice, and tumor volume was measured with a caliper (mm^3) over time (days). Only mice that developed a palpable tumor were included in the experimental cohorts.

that *cbl-b*$^{-/-}$ T cells cannot be anergized. (13, 17) Because some *cbl-b*$^{-/-}$ mice developed a late onset tumor (Fig. 1, A and B; and Fig. 3 B), we addressed the mechanism by which Cbl-b can confer spontaneous tumor rejection. To test whether the late onset of tumor growth in *cbl-b*$^{-/-}$ mice was the consequence of tumor-intrinsic "evasive" mechanisms rather than host-intrinsic "immunotolerance," we established cell lines from TC-1 tumors that showed late onset growth in *cbl-b*$^{-/-}$ mice. If tumor cells originating from *cbl-b*$^{-/-}$ mice trigger immunotolerance in a particular host mouse, then these tumors should again be rejected in *cbl-b*$^{-/-}$ mice that have survived a previous challenge of TC-1 cells (experienced *cbl-b*$^{-/-}$ mice). If tumors developed because of an escape mechanism intrinsic to the cancer cell, then these tumors should also progress when transferred into experienced *cbl-b*$^{-/-}$ mice. As a control, we also established tumors that grew in *cbl-b*$^{+/-}$ mice. As expected, tumors isolated from *cbl-b*$^{+/-}$ mice rapidly formed large tumors in wild-type mice, but were rejected when transferred into *cbl-b*$^{-/-}$ hosts (Fig. 5 A). Importantly, TC-1 tumors isolated from *cbl-b*$^{-/-}$ mice grew progressively in both *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ recipients. These results indicate that tumor growth in *cbl-b*$^{-/-}$ mice is a consequence of tumor escape rather than induction of immunotolerance.

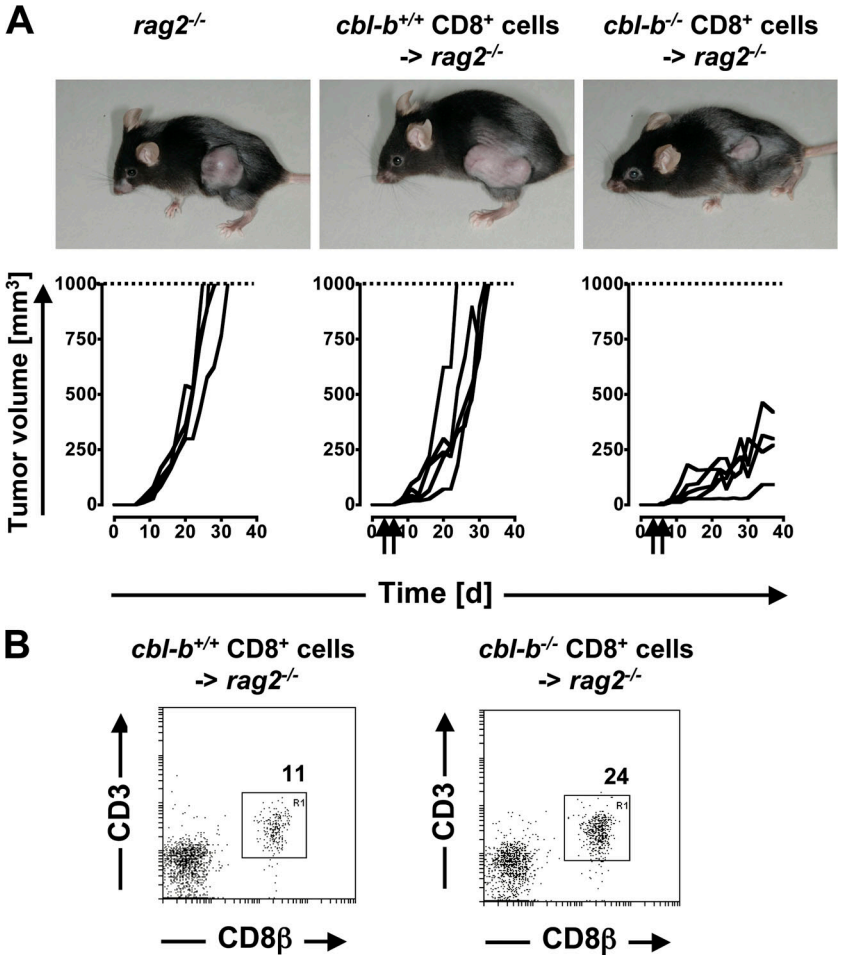

Figure 4. Therapeutic transfer of naive *cbl-b*$^{-/-}$ CD8$^+$ T cells is sufficient to mediate spontaneous rejection of established tumors. (A) *rag2*$^{-/-}$ mice were subcutaneously injected with 2.5 × 10^5 TC-1 cells. At day 3 and 6 after tumor cell injection, purified CD8$^+$ cells from naive *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice were adoptively transferred (i.v.) into the tumor-bearing *rag2*$^{-/-}$ mice (arrows). $n = 5$ per group. The *rag2*$^{-/-}$ control group ($n = 4$) received tumor cells, but no donor T cells. (top) Representative tumor sizes at the end of the experiment. (bottom) The kinetics of tumor growth. (B) Relative percentages of CD3$^+$CD8β$^+$ *cbl-b*$^{+/+}$ and CD3$^+$CD8β$^+$ *cbl-b*$^{-/-}$ T cells in the blood of adoptively transferred *rag2*$^{-/-}$ mice carrying TC-1 tumors. Representative flow cytometry data show CD3$^+$CD8β$^+$ T cell populations on day 7 after the second T cell transfer (day 13 after the first TC-1 injection).

cbl-b$^{-/-}$ mice carry long-lasting anticancer memory

Our data showed that CD8$^+$ T cells from *cbl-b* mutant mice can directly mediate and are sufficient for spontaneous tumor rejection. Moreover, mechanistically, we failed to observe induction of immunotolerance in TC-1 challenged *cbl-b*$^{-/-}$ mice. To expand these findings to additional therapeutic benefits of a potential T cell vaccine, we studied the antitumor memory response. To investigate whether aged *cbl-b* mutant mice, which rejected tumors and remained tumor free ("experienced *cbl-b*$^{-/-}$ mice"), have a long-lasting memory in response to the tumor, we rechallenged age-matched naive wild-type, naive *cbl-b*$^{-/-}$, and experienced *cbl-b*$^{-/-}$ mice (1 yr after the first challenge) with 100 times the lethal dose of TC-1 cells. At such a tumor cell concentration, all age-matched wild-type and *cbl-b*$^{+/-}$ control mice rapidly developed tumors (Fig. 5, C and D). Tumor development was abrogated in old naive *cbl-b*$^{-/-}$ mice, but all animals tested developed late onset tumors, indicating that the spontaneous antitumor response in *cbl-b*$^{-/-}$ is dependent on the tumor load. Intriguingly, 1 yr after the first challenge, we observed that experienced *cbl-b* knockout mice were able to reject the tumors even at the 100 times lethal tumor dose (Fig. 5 D). Moreover, 3 wk after tumor challenge, tumors appeared significantly smaller in the rechallenged experienced *cbl-b* knockout mice compared with naive knockout mice and naive heterozygous control mice (Fig. 5 C). It should be noted that incidence and severity of autoimmune organ infiltration were comparable between aged naive and TC-1–challenged *cbl-b* mutant mice at 1 yr after the first tumor challenge (not depicted), suggesting that even such long-lasting antitumor reactivity did not enhance the incidence or severity of autoimmunity (11, 12). Thus, even up to 1 yr after the first encounter with the tumor cells, *cbl-b*$^{-/-}$ mice carry an anticancer memory.

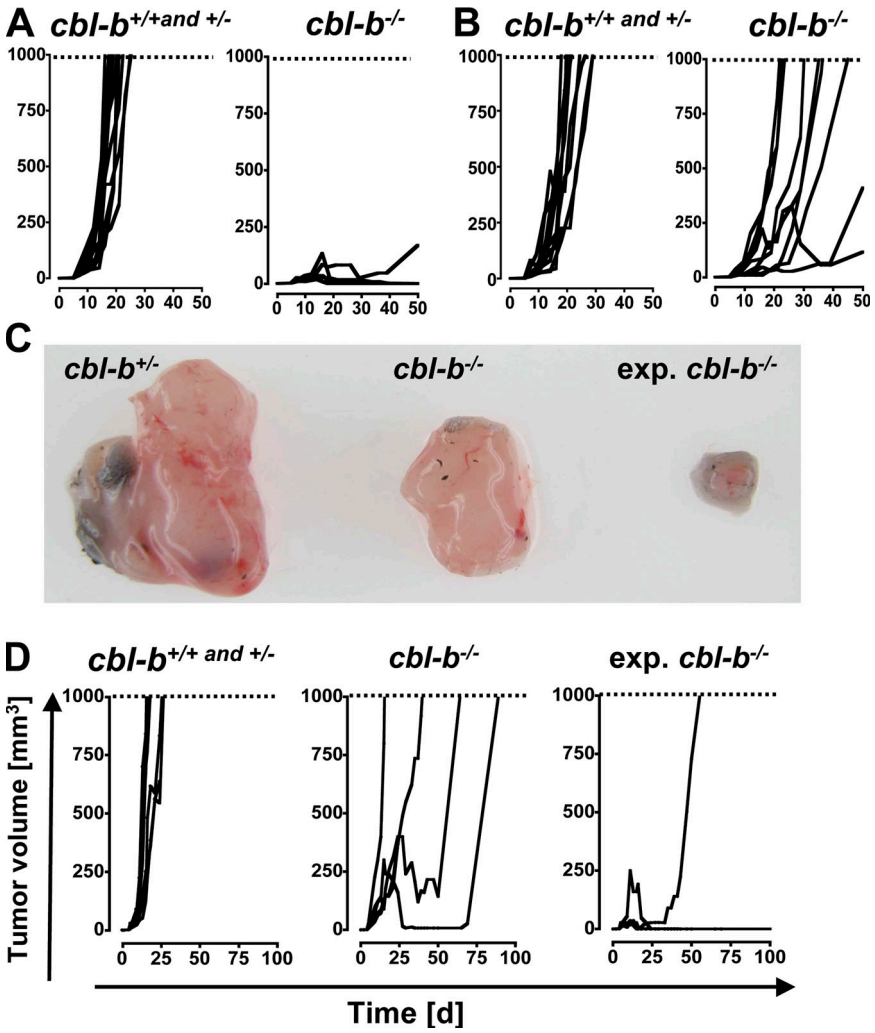

Figure 5. Tumor escape and long-lasting antitumor memory in *cbl-b*$^{-/-}$ mice. (A and B) Escape of *cbl-b*$^{-/-}$ mouse–derived TC-1 tumors. Tumor cell lines were generated from TC-1 tumors growing in *cbl-b*$^{+/-}$ mice (A) or generated from late-onset TC-1 tumors growing in *cbl-b*$^{-/-}$ mice (B). Tumor cells were injected (2.5×10^5) into 8–12-wk-old, naive, Cbl-b expressing control mice, and *cbl-b*$^{-/-}$ recipients. Kinetics of tumor growth was analyzed over the indicated time period. Graphs represent data from two pooled experiment of *cbl-b*$^{+/+}$ and *cbl-b*$^{+/-}$ ($n = 10$ for both cell lines) mice and *cbl-b*$^{-/-}$ mice ($n = 7$ for *cbl-b*$^{+/-}$-derived tumor cells; $n = 10$ for *cbl-b*$^{-/-}$-derived tumor cells). Of note, the kinetics of tumor growth was comparable between *cbl-b*$^{+/+}$ and *cbl-b*$^{+/-}$ mice. (C and D) Antitumor memory. *Cbl-b*$^{-/-}$ mice, which had received 2.5×10^5 TC-1 cells at 8–12 wk of age and stayed tumor free after rejection of the initial cancer, were kept under observation for 1 yr after TC-1 injection. These experienced (exp) *cbl-b*$^{-/-}$ mice, together with age-matched naive *cbl-b*$^{-/-}$ and age-matched naive *cbl-b*$^{+/+}$ and *cbl-b*$^{+/-}$ control mice, were rechallenged with a 10 times higher dose of TC-1 cells (2.5×10^6). (C) Appearance of representative tumors from each experimental cohort. Tumors were imaged 23 d after injection of 2.5×10^6 TC-1 cells. (D) Kinetics of TC-1 tumor cell growth in age-matched naive *cbl-b*$^{+/+}$ and *cbl-b*$^{+/-}$ control mice ($n = 5$), naive *cbl-b*$^{-/-}$ mice ($n = 6$), and experienced (exp) *cbl-b*$^{-/-}$ mice ($n = 6$). 2.5×10^6 TC-1 cells were injected into the flanks of 14-mo-old mice, and tumor volume was measured with a caliper (mm^3) over time (days). Of note, only mice that developed a palpable tumor were included in the experimental cohorts.

cbl-b$^{-/-}$ CD8$^+$ T cells are less sensitive to CD4$^+$CD25$^+$ regulatory T cell suppression

It has been reported that CD4$^+$CD25$^+$FoxP3$^+$ T-regulatory (T reg) cells suppress CD8$^+$ effector cell immunity in cancer (29). Moreover, T reg cells play a role in vaccination-mediated rejection of TC-1 tumors (30). Furthermore, it has been shown that *cbl-b*–deficient CD4$^+$ T cells are resistant to CD4$^+$CD25$^+$ T reg cell suppression (17). However, whether loss of Cbl-b also confers such resistance to CD8$^+$ T cells has never been established. Therefore, we first examined the numbers of T reg cells in TC-1 tumors from *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice. At 2 wk after TC-1 inoculation, the total numbers of CD4$^+$CD25$^+$ and CD8$^+$ tumor-infiltrating cells were similar in wild-type mice (Fig. 6 A). Tumors isolated from *cbl-b*$^{-/-}$ mice contained slightly increased (approximately twofold) numbers of infiltrating CD4$^+$CD25$^+$ cells. FoxP3 immunostaining of tumor-derived CD4$^+$CD25$^+$ cells showed that ~55% of these cells express FoxP3 both in

cbl-b$^{+/+}$ and *cbl-b*$^{-/-}$ mice (Fig. 6 A and Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20061699/DC1). The number of tumor-infiltrating CD8$^+$ cells was dramatically increased in TC-1–bearing *cbl-b*$^{-/-}$ mice compared with wild-type controls (Fig. 6 A). Of note, the total number of tumor cells and tumor sizes were comparable in the *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice analyzed (Fig. S3 B). These results show that T reg cells infiltrate TC-1 tumors in both *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice; however, loss of Cbl-b dramatically changes the ratio of CD8$^+$ T cells to T reg cells within the tumors.

We next examined whether loss of Cbl-b might change the function of T reg cells toward CD8$^+$ effector cell proliferation. Suppression of wild-type CD8$^+$ effector cells was comparable between *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ T reg cells (Fig. 6, B and D; and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20061699/DC1). In addition, similar to control CD4$^+$CD25$^+$ T reg cells, *cbl-b*$^{-/-}$ T reg cells did not proliferate upon anti-CD3 stimulation in vitro (Fig. S4). Thus, *cbl-b*$^{-/-}$ regulatory CD4$^+$CD25$^+$ T cells are bona fide suppressors toward wild-type and *cbl-b*$^{-/-}$ responder CD8$^+$ T cells, and loss of Cbl-b has no apparent effect on T reg cell functions. However, *cbl-b*–deficient effector CD8$^+$ T cells were resistant to suppression by wild-type, as well as *cbl-b*$^{-/-}$ T reg cells, at T reg cell/effector ratios that significantly suppressed proliferation of wild-type CD8$^+$ T cells. At a 2:1 T reg cell/CD8$^+$ effector cell ratio, however, we still observed suppression, albeit at lower levels, such as in *cbl-b*$^{-/-}$ effector CD8$^+$ T cells (Fig. 6, C and D; and Fig. S4). These data show that *cbl-b*$^{-/-}$ CD8$^+$ effector cells are resistant to T reg cell suppression.

cbl-b$^{-/-}$ mice show resistance to spontaneous UVB-induced skin cancer

Our results show that genetic ablation of Cbl-b confers spontaneous in vivo rejection of TC-1 tumor cells after subcutaneous inoculation. Therefore, we wanted to test whether Cbl-b also controls tumor resistance in a spontaneous tumor model relevant for human cancer, i.e., UVB-triggered skin cancer in mice. UVB irradiation is the most important risk factor for the induction of nonmelanoma skin cancer (31, 32). In addition, it has been shown that induction of immunosuppression by UVB is a skin cancer–promoting factor (31). To determine the role of Cbl-b in the generation of UVB-induced cutaneous malignancies, we chronically irradiated cohorts of *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice with UVB on their shaved backs. Tumor development was recorded over time (Fig. 7 A and Fig. S5, A and B, available at http://www.jem.org/cgi/content/full/jem.20061699/DC1). In both *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice, the first visible progressively growing tumors appeared ~300 d after the initial UVB treatment, suggesting that tumor onset is comparable between control and *cbl-b* mutant animals. Most UV-induced skin tumors were located on the ears and backs of the mice (Fig. 7 C). However, *cbl-b*$^{-/-}$

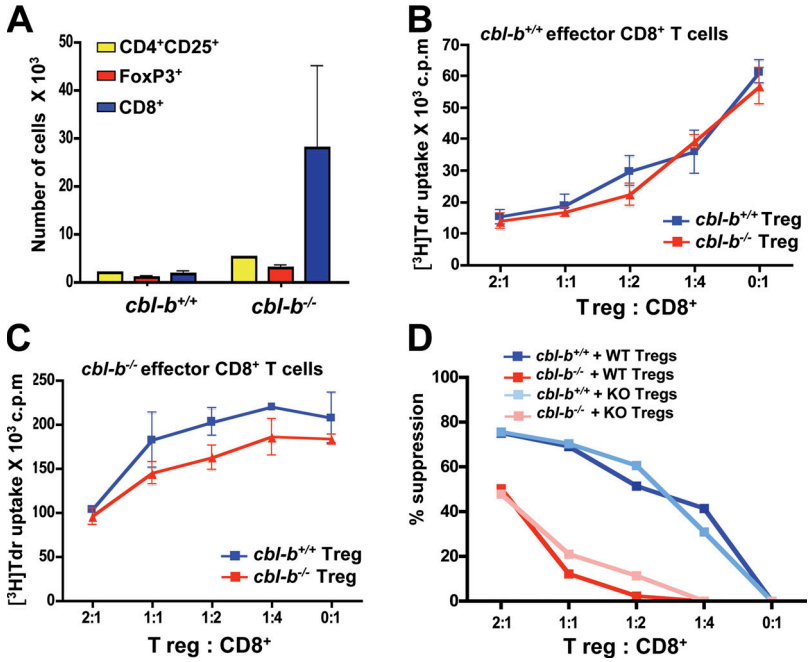

Figure 6. *cbl-b*$^{-/-}$ effector CD8$^+$ T cells are resistant to CD4$^+$CD25$^+$ T reg cell suppression. (A) Numbers of tumor infiltration for CD4$^+$CD25$^+$, FoxP3$^+$, and CD8$^+$ cells per 10^6 tumor cells in *cbl-b*$^{-/-}$ ($n = 4$ tumors) and *cbl-b*$^{+/+}$ ($n = 6$ tumors) mice. Data are shown as the mean ± the SEM. Proliferation of wild-type CD8$^+$ effector T cells (B) and proliferation of *cbl-b*$^{-/-}$ CD8$^+$ effector T cells in the presence of *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ CD4$^+$CD25$^+$ T reg cells at various T reg cell/Teff concentrations (C). Proliferation in B and C was measured by [^{3}H]thymidine incorporation for the last 12 h of a 72-h stimulation with anti-CD3ε. Data (mean values of a triplicate culture ± the SEM) are from one out of three different experiments with similar results. (D) Percent suppression of proliferation of CD8$^+$ effector T cells from *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice by *cbl-b*$^{+/+}$ (WT) and *cbl-b*$^{-/-}$ (KO) T reg cells at various T reg cell/Teff concentrations.

mice exhibited markedly reduced susceptibility to photocarcinogenesis compared with wild-type mice, whereas 15 out of 18 wild-type mice developed 2–3 visible tumors each, only 6 out of 21 *cbl-b*$^{-/-}$ mice developed visible skin cancer, and in only one case did we observe more than one tumor per *cbl-b*$^{-/-}$ mouse (Fig. 7 A).

Intriguingly, whereas in *cbl-b*$^{+/+}$ mice skin tumors grew progressively in all cases observed (Fig. S5 A), the initial phase of tumor growth in *cbl-b*$^{-/-}$ mice was followed by a marked reduction in tumor mass (Fig. 7 C and Fig. S5 B). Note that one *cbl-b*$^{-/-}$ mouse was killed for histology, therefore tumor progression or reduction could not be followed (Fig. S5 B).

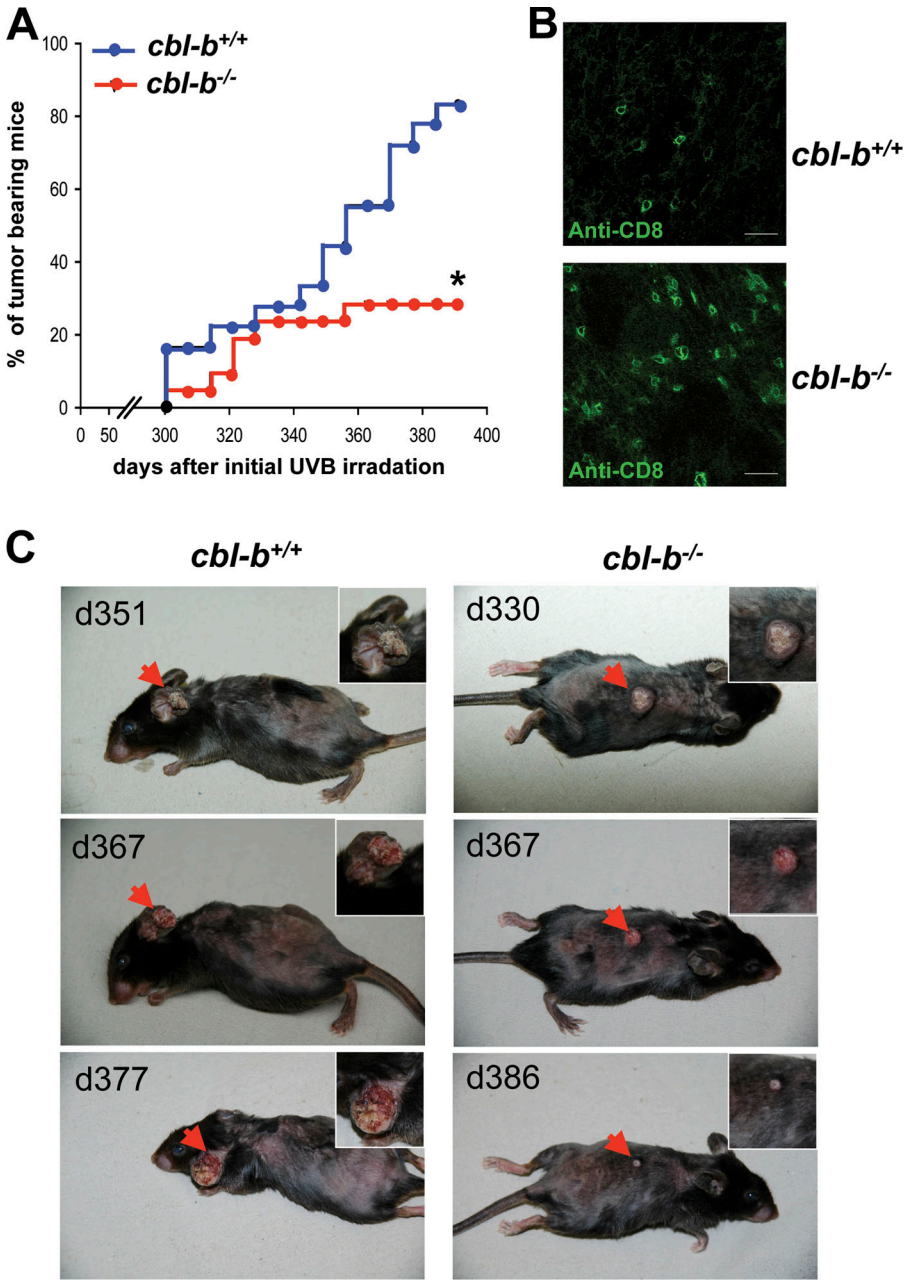

Figure 7. *cbl-b*$^{-/-}$ mice show significantly decreased susceptibility to spontaneous UVB-induced skin cancer. (A) Kaplan-Meier curves of tumor-bearing *cbl-b*$^{+/+}$ ($n = 18$) and *cbl-b*$^{-/-}$ ($n = 21$) mice during chronic UVB irradiation. Tumor incidence in the *cbl-b*$^{-/-}$ cohort was significantly reduced compared with *cbl-b*$^{+/+}$ mice (*, $P < 0.05$ from day 363 and $P < 0.001$ from day 377 onward; log-rank test). (B) Confocal images of *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ skin tumor sections stained for CD8$^+$ cells by immunofluorescence. Bars, 25 μm. (C) Representative UVB-induced tumor growth in one wild-type (left) and one *cbl-b*$^{-/-}$ mouse (right). Tumor growth is shown over time in the same two mice (days after first UVB irradiation is indicated) Note the progressive reduction of tumor mass in the *cbl-b*$^{-/-}$ mouse. Insets are higher magnifications of tumors. Histology confirmed epithelial origin of the tumors in control and *cbl-b*$^{-/-}$ mice.

In line with our TC-1 data, the number of tumor-infiltrating $CD8^+$ T cells was dramatically increased in the UVB-induced skin tumors of *cbl-b*$^{-/-}$ mice, as determined by immunofluorescence (Fig. 7 B). Furthermore, using TUNEL staining, we detected increased numbers of apoptotic cells in skin tumors of *cbl-b*$^{-/-}$ mice compared with *cbl-b*$^{+/+}$ skin tumors (Fig. S6 A, available at http://www.jem.org/cgi/content/full/jem.20061699/DC1). Numbers and ratios of T cells, as well as expression of surface markers (CD44, CD43, CD69, CD28, CTLA4, CD3, CD8, CD4, CD127, CD62L, CD25, and FoxP3) in the draining lymph nodes, were comparable among tumor-bearing *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice (not depicted). To address whether, similar to our TC-1 tumor model, $CD8^+$ T cells are also the critical cell type involved in the surveillance of UVB-induced skin cancer, $CD8^+$ cells were depleted in UVB-treated *cbl-b*$^{-/-}$ mice that had received UVB irradiation but never developed a tumor. Remarkably, only 10 d after starting the depletion by injection of the CD8-depleting antibody (Fig. S6 B), 50% of UVB-treated (and previously cancer free) *cbl-b*$^{-/-}$ mice ($n = 4$) developed rapidly growing tumors, whereas all IgG isotype control-treated, UVB-treated *cbl-b*$^{-/-}$ mice ($n = 4$) remained tumor free (Fig. 8, A and B). In conclusion, our results show that *cbl-b*–deficient mice exhibit reduced skin cancer and are able to reject spontaneous, UVB-induced skin tumors.

DISCUSSION

Various schemes for immunological treatment of tumors have been described, including genetic alterations of tumors with cytokines or costimulatory molecules, or the generation of tumor-specific cytotoxic T cells (33–35). However, immunotherapy is still difficult because most tumors are insufficiently recognized, do not elicit a robust immune response, or induce immunotolerance (4, 5, 36). Several attempts have been made to break tumor tolerance and enhance tumor immunity using transgenic models, transplantation of T cells or dendritic cells, or novel vaccination regimens against known tumor antigens (8, 9). Moreover, many immunotherapies are limited because of severe side-effects and the availability of tumor-reactive immune cells and combination therapies (7, 37).

Our results show that inactivation of a single negative regulator of T cell signaling confers anticancer activity in vivo using two distinct tumor models relevant for human cancers. This antitumor activity occurs spontaneously, and tumor growth is completely eradicated in virtually all *cbl-b* mutant mice in the TC-1 tumor model, as well as in our

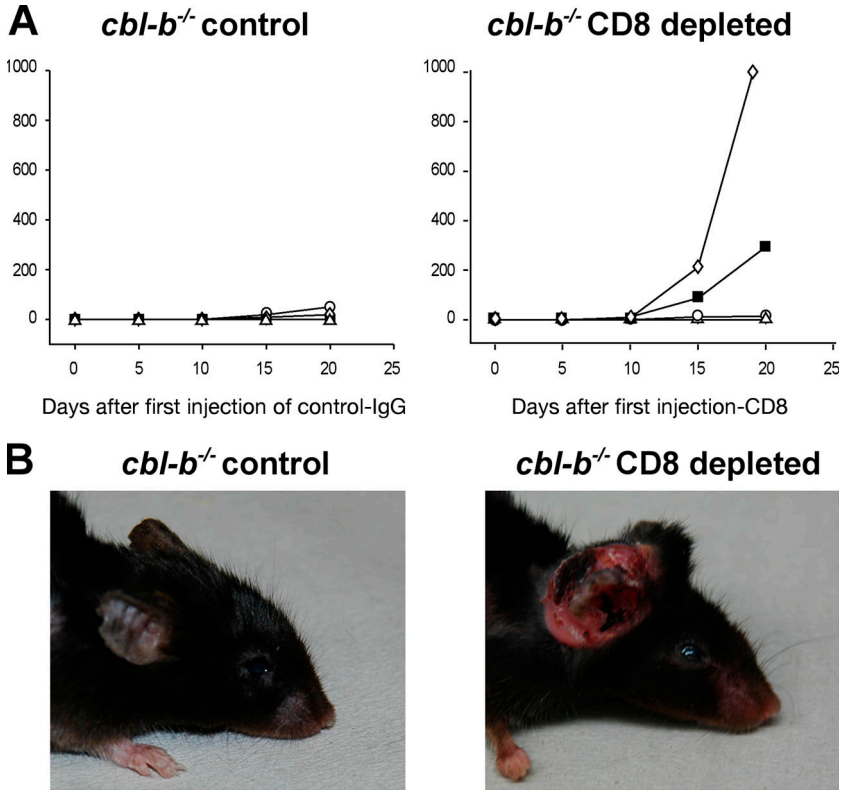

Figure 8. Depletion of $CD8^+$ cells in UVB-treated *cbl-b*$^{-/-}$ mice leads to rapid tumor outgrowth. (A) Kinetics of progressive tumor growth inindividual $CD8^+$ cell–depleted, UVB-treated *cbl-b*$^{-/-}$ ($n = 4$), and IgG control-treated UVB-treated *cbl-b*$^{-/-}$ ($n = 4$) mice. Depletion was performed 130 d after the last UVB treatment. Only mice that received UVB treatment for 9 mo, but did not develop skin cancer, were included in this experiment. Tumor volume in millimeters^3 was measured over a period of days. Shapes represent individual mice. (B) Representative UVB-induced tumor growth in one IgG-treated *cbl-b*$^{-/-}$ mouse (left) and one $CD8^+$ cell–depleted *cbl-b*$^{-/-}$ mouse (right) imaged 22 d after initial depletion.

spontaneous UVB-induced skin cancer model. In the TC-1 model, we could show that antitumor memory is maintained for >1 yr in *cbl-b*$^{-/-}$ mice. Thus, we have identified a dominant "tolerogenic" factor that actively represses activation of tumor-specific T cells in vivo. Although we explored the role of Cbl-b in two distinct tumor models, further studies are required to determine whether Cbl-b is, indeed, a key molecule that confers antitumor immunity in additional cancer types, including tumor models with defined high or low immunogenicity. Moreover, it will be interesting to explore whether *cbl-b*$^{-/-}$ CD8$^+$ T cells cooperate with other cell types in tumor rejection.

Mechanistically, deletion of *cbl-b* might affect anticancer immunity at several levels. One rate-limiting factor for successful antitumor immunity is the induction of T reg cells in cancer. Interestingly, whereas loss of Cbl-b does not affect T reg cell-mediated suppressor functions toward CD8$^+$ T cells, *cbl-b*$^{-/-}$ CD8$^+$ effector T cells display resistance to proliferative suppression. Thus, similar to previous reports that Cbl-b may regulate suppression of CD4$^+$ effector cells (17, 18), we have identified a novel function of Cbl-b in T reg cell–mediated suppression of effector CD8$^+$ T cells. Our results also indicate that Cbl-b must regulate additional mechanisms involved in tumor rejection by CD8$^+$ T cells. For instance, the expansion and proliferation of CD8$^+$ T cells is increased in *cbl-b*$^{-/-}$ mice, and we observed a rapid onset and elevated numbers of tumor-infiltrating effector CD8$^+$ T cells in TC-1 tumors, as well as our spontaneous skin cancer model. These results would be in line with enhanced penetration of T cells into the tumors, possibly as a result of enhanced activation. Moreover, our preliminary data suggest that CD8$^+$ T cells from *cbl-b*$^{-/-}$ mice exhibit increased sensitivity to dendritic cells loaded with TC-1–derived tumor antigens. We propose that Cbl-b affects multiple regulatory circuits in antitumor immunity.

Importantly, established TC-1 tumors can be treated by the transfer of nontransgenic, "naïve" CD8$^+$ *cbl-b*$^{-/-}$ T cells that have previously never encountered the tumor antigen. Loss of Cbl-b in the CD8$^+$ compartment alone is both necessary and sufficient to induce potent antitumor immunity, thereby perhaps providing a direct means of targeting tumors via CD8$^+$ T cell responses even in the context of ineffective costimulation, impaired CD4$^+$ T cell help, or T reg cell immunosuppression. Thus, inactivation of Cbl-b might be a potent new strategy for anticancer immunotherapy on multiple levels to augment the effectiveness of tumor-specific CD8$^+$ T cells in humans.

MATERIALS AND METHODS

Mice. *cbl-b* mutant mice have been previously described (11) and were crossed onto a C57BL/6 background for >10 generations. C57BL/6 wild-type mice and *rag2*$^{-/-}$ mice were obtained from our in-house breeding stock. *cbl-b*$^{+/-}$ littermates showed the same results as wild-type mice. Only female mice were used in all experiments because TC-1 cells are derived from female mice. All mice were maintained under specific pathogen-free conditions and used in accordance with institutional guidelines (permission was obtained from Magistrat 58 of the City of Vienna).

Tumor cells, dendritic cell culture, antibodies, and peptides. TC-1 cells have been previously reported and were generated by cotransformations of primary C57BL/6 mouse lung fibroblasts with an activated c-H-*ras* oncogene and the HPV-16 E6 and E7 oncoproteins (19). TC-1 cells were maintained in IMDM medium containing 10% FCS, 100 IU/ml penicillin, and 2 mM glutamine, supplemented with 0.5 mg/ml G418, 1 mM Na pyruvate, and 30 μM 2-ME. The HPV-16–derived and H2D^b-restricted peptide E7$^{49–57}$ (RAHYNIVTF) was used for all restimulation experiments (23). Antibodies against mouse CD3ε (clone 145-2C11), CD4 (RM4-5), CD8β.2 (53–5.8), CD8α (53–6.7), TCR-β (H57-597), CD11b (M1/70), CD11c (HL3), CD16/32 (2.4G2), GR1 (RB6-8C5), NK1.1 (PK136), IFNγ (XMG1.2), CD44 (IM7), CD62L (Mel-14), CD25 (PC61), CTLA-4 (UC10-4F10-11), CD43 (1B11), CD69 (H1.2F3), CD28 (37.51), and CD127 (clone SB/199) were purchased from BD Biosciences. The anti-Ki67 antibody was purchased from Novocastra. The anti–mouse FoxP3 antibody (clone FJK-16s) was obtained from eBioscience. Hybridomas for production of the depleting antibodies to CD4 (clone GK1.5) and CD8 (clone 2.43) were grown in serum-free medium and purified using a protein A/G column. Cells were analyzed by four-color flow cytometry on a cytometer (FACScalibur; Becton Dickinson) using CellQuest software (BD Biosciences).

In vivo TC-1 tumor cell growth. TC-1 cells were injected s.c. into the shaved left flank of 8–12-wk-old female mice. In all experiments at day zero, 2.5×10^5 TC-1 tumor cells were injected s.c., whereas for tumor memory experiments 2.5×10^6 TC-1 tumor cells were s.c. injected. CD8$^+$ cells or CD4$^+$ cells were depleted by injection of 50 μg of depletion antibodies i.p. per mouse at day 4 and 2 before tumor inoculation (day 0). At day 1, complete depletion of respective cell subsets was confirmed by FACS analysis. The depletion was repeated weekly throughout the experiment. In all experimental cohorts, tumor growth was monitored three times per week by measuring tumor length, width, and height with a caliper. Mice were killed when tumor volume reached 1 cm^3. For tumor escape/immunotolerance experiments, tumors were isolated from *cbl-b*$^{+/-}$ or *cbl-b*$^{-/-}$ mice. Cells were passaged in vitro for 1 mo, and aliquots were kept. Before use for experiments, cells were passaged six times. These newly established TC-1 tumor cell lines cells were then injected into 8–12-wk-old naive *cbl-b*$^{+/+}$ or *cbl-b*$^{-/-}$ recipients, and tumor growth was analyzed as described. All experimental procedures performed on mice were in accordance with institutional guidelines.

UVB-induced photocarcinogenesis. Within the solar spectrum, the UVB range (290–320 nm) is responsible for carcinogenesis and immunosuppression. Therefore, a bank of four UVB TL40W/12 (Philips) sunlamps was used, which have an emission spectrum from 280 to 350 nm, with a peak at 306 nm. These lamps deliver an average dose of 8 W/m^2, as measured with an IL-1700 UV detector and a SED 24 filter (both from International Light). The mice were placed on a shelf 20 cm below the light bulbs for irradiation. The cage order was systematically rotated before each treatment to compensate for uneven lamp output along the shelf, as previously described (38, 39). Female mice were shaved with electric clippers once per week on the entire dorsum. Beginning at 10 wk of age, mice were irradiated three times per week with 2.5 kJ/m^2 for the first 4 wk, followed by 5 kJ/m^2 for 4 wk, 7.5 kJ/m^2 for 4 wk, and, finally, 10 kJ/m^2 for 6 mo. All mice were monitored weekly for tumor development by two independent investigators for an additional 4 mo. The location and growth of each tumor >2 mm in diameter was recorded. The method of Kaplan and Meier was used to describe the probability of tumor development in the carcinogenesis study. Immunohistochemistry of UV-induced tumors was performed on 3-μm cryostat sections fixed in acetone according to standard methods. Slides were incubated in the appropriate dilution of anti-CD8 (clone 53–6.7; Becton Dickinson) and an Alexa Fluor 488–labeled secondary antibody (Invitrogen) and examined using a 200M confocal microscope and LSM510 Meta software (both from Carl Zeiss MicroImaging, Inc.). Statistical differences for the development of tumors between the two strains of mice were determined using a log-rank test.

Therapeutic CD8$^+$ T cell transfer into *rag2*$^{-/-}$ mice. CD8$^+$ cells were purified from spleens and lymph nodes of naive *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice by positive selection using Magnetic beads against CD8 (Miltenyi Biotec) following the manufacturer's recommendations (purity was over 90% as determined by CD8β and CD3 double staining). 3 and 6 d after tumor cell injection 3×10^6 and 2×10^6 CD8$^+$ cells were adoptively transferred into the tumor-bearing *rag2*$^{-/-}$ mice, respectively. Tumor growth was monitored three times per week.

Restimulation and intracellular cytokine staining. Cells were isolated from spleens and lymph nodes of tumor-bearing *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice, as well as naive controls of each genotype, and CD8$^+$ T cells were purified using positive magnetic bead sorting (Miltenyi Biotec). CD8$^+$ cells (2×10^5/well) were stimulated in vitro for 3 d with HPV-16–E7$^{49–57}$ peptide-loaded splenocytes (10 μg/ml peptide; 20 Gy irradiated; 2×10^5 APCs per well) in complete medium. Golgi plug was added for the last 4 h, and the cells were stained for CD8β and CD3 surface expression. After fixation, intracellular IFNγ levels were determined using a Cytoperm/Cytofix kit from BD Biosciences (according to the manufacturer's protocol). Cells were analyzed by FACS (Becton Dickinson).

Histology and immunohistochemistry. Tumors were dissected and frozen in optimal cutting temperature compound for cryosections or fixed with 3.7% formaldehyde before paraffin embedding. For histological analysis, 5-μm-thick sections were cut and stained with hematoxylin and eosin using standard protocols. TUNEL (Roche) and Ki67 stainings were performed on paraffin-embedded sections. Tumor-infiltrating CD8β$^+$ and CD4$^+$ were detected on frozen sections by immunofluorescence. FoxP3 staining was performed on frozen sections and on PolyPrep slides (Sigma-Aldrich) coated with FACS-sorted CD4$^+$CD25$^+$ cells by immunocytochemistry. Confocal images were taken using a LSM510 microscope.

T reg cell suppressor assays. Different concentrations of sorted CD25$^+$CD4$^+$ T reg cell cells from naive *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ mice were co-cultured with CD8$^+$ effector T cells (5×10^4/well) isolated from wild-type and *cbl-b*$^{-/-}$ mice in the presence of 2×10^5 irradiated (3,000 rads), T-depleted splenocytes, and 1 μg/ml of purified mouse anti-CD3ε in 200 μl IMDM supplemented with 10% FCS in 96-well, round-bottomed plates. Proliferation was measured by scintillation counting after a pulse with 1 μCi of [^{3}H]thymidine per well for the last 12 h of a 72-h incubation period.

Online supplemental materials. Fig. S1 shows the CD8$^+$ cell infiltration into TC-1 tumors 8 d after tumor inoculation. Fig S2 shows the depletion efficiency of CD4$^+$ and CD8$^+$ T cell subsets by depletion antibodies. Fig. S3 shows the percentages of infiltrating FoxP3$^+$ cells among CD4$^+$CD25$^+$ cells isolated from TC1 tumors 14 d after tumor cell inoculation detected by immunostaining, and the absolute cell numbers in tumors isolated from tumors 14 d after TC-1 inoculation. Fig. S4 shows the proliferation of CD8$^+$ T cells isolated from wild-type and *cbl-b*$^{-/-}$ mice in the presence or absence of T reg cells from both genotypes and under various conditions. Fig. S5 shows graphs of the tumor growth in individual UVB-treated *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice. Fig. S6 shows TUNEL and CD8 staining of UVB-induced skin tumors from *cbl-b*$^{-/-}$ and *cbl-b*$^{+/+}$ mice, as well as the efficacy of CD8 depletion in UVB-treated *cbl-b*$^{-/-}$ mice and UVB-treated, nondepleted *cbl-b*$^{-/-}$ control mice.

We thank Vukoslav Komnenovic, Ivan Botto, Johannes Tkadletz, Gabi Stengl, and Pawel Pasierbek for technical support, and all members of the Penninger laboratory for helpful discussions.

J.M. Penninger is supported by grants from Euro-Thymaide, the Austrian Science Fund (Spezialforschungbereich on immunotolerance), Innovative Mouse Models for Functional Genomics in Immunology, Euro-RA, the Austrian National Bank, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, and the Austrian Ministry of Sciences. S. Loeser is a Euro-Thymaide fellow. K. Loser and S. Besissert are supported by the Interdisciplinary Center of Clinical Research (grants Lo2/65/04 and Lo2/17/07), the Innovative Medical Research Fund grant Lo110603, and the German Research Association grant BE1580/7-1. M.S. Bijker is supported by the Dutch Cancer Society grant UL 2003-2817. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20061699/DC1.

J.M. Penninger has shares in a company that attempts to develop a Cbl-b inhibitor. The authors have no other conflicting financial interests.

Submitted: 8 August 2006
Accepted: 1 March 2007

REFERENCES

1. Pardoll, D.M. 1993. New strategies for enhancing the immunogenicity of tumors. *Curr. Opin. Immunol.* 5:719–725.
2. Burnet, F.M. 1970. The concept of immunological surveillance. *Prog. Exp. Tumor Res.* 13:1–27.
3. Dunn, G.P., L.J. Old, and R.D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity.* 21:137–148.
4. Staveley-O'Carroll, K., E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, and H. Levitsky. 1998. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc. Natl. Acad. Sci. USA.* 95:1178–1183.
5. Speiser, D.E., R. Miranda, A. Zakarian, M.F. Bachmann, K. McKall-Faienza, B. Odermatt, D. Hanahan, R.M. Zinkernagel, and P.S. Ohashi. 1997. Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J. Exp. Med.* 186:645–653.
6. Yu, X., R. Abe, and R.J. Hodes. 1998. The role of B7-CD28 costimulation in tumor rejection. *Int. Immunol.* 10:791–797.
7. Phan, G.Q., J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, et al. 2003. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. USA.* 100:8372–8377.
8. Lollini, P.L., F. Cavallo, P. Nanni, and G. Forni. 2006. Vaccines for tumour prevention. *Nat. Rev. Cancer.* 6:204–216.
9. Townsend, S.E., and J.P. Allison. 1993. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science.* 259:368–370.
10. Thien, C.B., and W.Y. Langdon. 2001. Cbl: many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* 2:294–307.
11. Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y.Y. Kong, T. Sasaki, A. Oliveira-dos-Santos, S. Mariathasan, D. Bouchard, A. Wakeham, A. Itie, et al. 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature.* 403:211–216.
12. Chiang, Y.J., H.K. Kole, K. Brown, M. Naramura, S. Fukuhara, R.J. Hu, I.K. Jang, J.S. Gutkind, E. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature.* 403: 216–220.
13. Jeon, M.S., A. Atfield, K. Venuprasad, C. Krawczyk, R. Sarao, C. Elly, C. Yang, S. Arya, K. Bachmaier, L. Su, et al. 2004. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity.* 21: 167–177.
14. Heissmeyer, V., F. Macian, S.H. Im, R. Varma, S. Feske, K. Venuprasad, H. Gu, Y.C. Liu, M.L. Dustin, and A. Rao. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* 5:255–265.
15. Krawczyk, C.M., R.G. Jones, A. Atfield, K. Bachmaier, S. Arya, B. Odermatt, P.S. Ohashi, and J.M. Penninger. 2005. Differential control of CD28-regulated in vivo immunity by the E3 ligase Cbl-b. *J. Immunol.* 174:1472–1478.
16. Gronski, M.A., J.M. Boulter, D. Moskophidis, L.T. Nguyen, K. Holmberg, A.R. Elford, E.K. Deenick, H.O. Kim, J.M. Penninger, B. Odermatt, et al. 2004. TCR affinity and negative regulation limit autoimmunity. *Nat. Med.* 10:1234–1239.
17. Wohlfert, E.A., M.K. Callahan, and R.B. Clark. 2004. Resistance to CD4+CD25+ regulatory T cells and TGF-beta in Cbl-b−/− mice. *J. Immunol.* 173:1059–1065.

18. Wohlfert, E.A., L. Gorelik, R. Mittler, R.A. Flavell, and R.B. Clark. 2006. Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional defect in T cell TGF-beta sensitivity in vitro and in vivo. *J. Immunol.* 176:1316–1320.
19. Lin, K.Y., F.G. Guarnieri, K.F. Staveley-O'Carroll, H.I. Levitsky, J.T. August, D.M. Pardoll, and T.C. Wu. 1996. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res.* 56:21–26.
20. zur Hausen, H. 1996. Papillomavirus infections–a major cause of human cancers. *Biochim. Biophys. Acta.* 1288:F55–F78.
21. Harper, D.M., E.L. Franco, C. Wheeler, D.G. Ferris, D. Jenkins, A. Schuind, T. Zahaf, B. Innis, P. Naud, N.S. De Carvalho, et al. 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet.* 364:1757–1765.
22. Zwaveling, S., S.C. Ferreira Mota, J. Nouta, M. Johnson, G.B. Lipford, R. Offringa, S.H. van der Burg, and C.J. Melief. 2002. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J. Immunol.* 169:350–358.
23. Feltkamp, M.C., H.L. Smits, M.P. Vierboom, R.P. Minnaar, B.M. de Jongh, J.W. Drijfhout, J. ter Schegget, C.J. Melief, and W.M. Kast. 1993. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur. J. Immunol.* 23:2242–2249.
24. Diehl, L., A.T. den Boer, S.P. Schoenberger, E.I. van der Voort, T.N. Schumacher, C.J. Melief, R. Offringa, and R.E. Toes. 1999. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat. Med.* 5:774–779.
25. Lavergne, E., C. Combadiere, M. Iga, A. Boissonnas, O. Bonduelle, M. Maho, P. Debre, and B. Combadiere. 2004. Intratumoral CC chemokine ligand 5 overexpression delays tumor growth and increases tumor cell infiltration. *J. Immunol.* 173:3755–3762.
26. Cavallo, F., R. Offringa, S.H. van der Burg, G. Forni, and C.J. Melief. 2006. Vaccination for treatment and prevention of cancer in animal models. *Adv. Immunol.* 90:175–213.
27. Melief, C.J., R.E. Toes, J.P. Medema, S.H. van der Burg, F. Ossendorp, and R. Offringa. 2000. Strategies for immunotherapy of cancer. *Adv. Immunol.* 75:235–282.
28. Willimsky, G., and T. Blankenstein. 2005. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature.* 437: 141–146.
29. Turk, M.J., J.A. Guevara-Patino, G.A. Rizzuto, M.E. Engelhorn, S. Sakaguchi, and A.N. Houghton. 2004. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J. Exp. Med.* 200:771–782.
30. Di Paolo, N.C., S. Tuve, S. Ni, K.E. Hellstrom, I. Hellstrom, and A. Lieber. 2006. Effect of adenovirus-mediated heat shock protein expression and oncolysis in combination with low-dose cyclophosphamide treatment on antitumor immune responses. *Cancer Res.* 66:960–969.
31. Beissert, S. 2002. Use of mutant mice in photoimmunological and photocarcinogenic investigations. *Methods.* 28:130–137.
32. Woodhead, A.D., R.B. Setlow, and M. Tanaka. 1999. Environmental factors in nonmelanoma and melanoma skin cancer. *J. Epidemiol.* 9: S102–S114.
33. Gattinoni, L., D.J. Powell Jr., S.A. Rosenberg, and N.P. Restifo. 2006. Adoptive immunotherapy for cancer: building on success. *Nat. Rev. Immunol.* 6:383–393.
34. Pardoll, D. 2003. Does the immune system see tumors as foreign or self? *Annu. Rev. Immunol.* 21:807–839.
35. Figdor, C.G., I.J. de Vries, W.J. Lesterhuis, and C.J. Melief. 2004. Dendritic cell immunotherapy: mapping the way. *Nat. Med.* 10:475–480.
36. Wick, M., P. Dubey, H. Koeppen, C.T. Siegel, P.E. Fields, L. Chen, J.A. Bluestone, and H. Schreiber. 1997. Antigenic cancer cells grow progressively in immune hosts without evidence for T cell exhaustion or systemic anergy. *J. Exp. Med.* 186:229–238.
37. Dudley, M.E., J.R. Wunderlich, P.F. Robbins, J.C. Yang, P. Hwu, D.J. Schwartzentruber, S.L. Topalian, R. Sherry, N.P. Restifo, A.M. Hubicki, et al. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science.* 298: 850–854.
38. Kripke, M.L. 1977. Latency, histology, and antigenicity of tumors induced by ultraviolet light in three inbred mouse strains. *Cancer Res.* 37:1395–1400.
39. Beissert, S., J.A. Bluestone, I. Mindt, M. Voskort, D. Metze, A. Mehling, T.A. Luger, T. Schwarz, and S. Grabbe. 1999. Reduced ultraviolet-induced carcinogenesis in mice with a functional disruption in B7-mediated costimulation. *J. Immunol.* 163:6725–6731.