The deubiquitinase Otub1 controls the activation of CD8+ T cells and NK cells by regulating IL-15-mediated priming

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CD8+ T cells and natural killer (NK) cells are central cellular components of immune responses against pathogens and cancer, which rely on interleukin (IL)-15 for homeostasis. Here we show that IL-15 also mediates homeostatic priming of CD8+ T cells for antigen-stimulated activation, which is controlled by a deubiquitinase, Otub1. IL-15 mediates membrane recruitment of Otub1, which inhibits ubiquitin-dependent activation of AKT, a kinase that is pivotal for T cell activation and metabolism. Otub1 deficiency in mice causes aberrant responses of CD8+ T cells to IL-15, rendering naive CD8+ T cells hypersensitive to antigen stimulation characterized by enhanced metabolic reprogramming and effector functions. Otub1 also controls the maturation and activation of NK cells. Deletion of Otub1 profoundly enhances anticancer immunity by unleashing the activity of CD8+ T cells and NK cells. These findings suggest that Otub1 controls the activation of CD8+ T cells and NK cells by functioning as a checkpoint of IL-15-mediated priming.

CD8+ T cells and NK cells are major cytotoxic effector cells of the immune system responsible for the destruction of pathogen-infected cells and cancer cells1–4. CD8+ T cells detect specific antigens via the T cell antigen receptor (TCR), whereas NK cells are innate lymphocytes that use different receptors for sensing target cells. These effector cells also function in different phases of an immune response, with NK cells acting in the early phase of innate immunity and CD8+ T cells acting in the late phase of adaptive immunity. NK cells also play an important role in regulating T cell responses5. Thus, CD8+ T cells and NK cells are considered complementary cytotoxic effectors and have been actively explored for cancer immunotherapy4.

A common feature of CD8+ T cells and NK cells is their dependence on the cytokine IL-15 for homeostasis6–8. IL-15 is a member of the common γ-chain (γc) family of cytokines that function via the IL-15 receptor (IL-15R) complex, composed of IL-15Rα, IL-15Rβ (also called IL-2Rβ or CD122) and γc (also called CD132). IL-15 induces signaling via a transpresentation mechanism, in which IL-15Rβ binds to IL-15 and transpresents IL-15 to the IL-15R β–γ complex on responding cells6. Under physiological conditions, IL-15 is specifically required for the homeostasis of CD8+ T cells and NK cells that express high levels of IL-15R β–γ, heterodimer7–9. Exogenously administered IL-15 can also promote activation of CD8+ T cells and NK cells and has therefore been exploited as an adjuvant for cancer immunotherapies10–12. However, the physiological function of IL-15 in regulating the activation of CD8+ T cells and NK cells is poorly defined and how signal transduction from IL-15R is regulated is also unknown.

Ubiquitination is a crucial mechanism that regulates diverse biological processes, including immune responses13. Ubiquitination is a reversible reaction counter-regulated by ubiquitinating enzymes and deubiquitinases (DUBs)14. In vitro studies identified an atypical DUB, Otub1, which can both directly cleave ubiquitin chains from target proteins and indirectly inhibit ubiquitination by blocking the function of specific ubiquitin-conjugating enzymes (E2s), including the K63-specific E2 Ubc13 (refs. 14–17). However, the in vivo physiological function of Otub1 has been poorly defined. In the present study, we identified Otub1 as a pivotal regulator of IL-15R signaling and homeostasis of CD8+ T cells and NK cells. Otub1 controls IL-15-stimulated activation of AKT, a pivotal kinase for T cell activation, metabolism and effector functions18–20. Our results suggest that Otub1 also controls the activation and function of CD8+ T cells and NK cells in immune responses against infections and cancer.

Results

T cell-specific Otub1 deficiency causes aberrant activation of CD8+ T cells. To study the function of Otub1 in T cells, we generated mice with conditional knockout of Otub1 in T cells (TKO mice; Supplementary Fig. 1a–c). The Otub1-TKO mice had normal frequencies of thymocyte and peripheral T cell populations (Supplementary Fig. 1d,e). However, they had increased frequencies of effector/memory-like (CD44hi) CD8+ T cells producing the effector cytokines interferon (IFN)-γ, tumor necrosis factor (TNF) and IL-2 (Fig. 1a,b). Although Otub1 was similarly expressed in CD4+ and CD8+ T cells (data not shown), Otub1 deficiency did not increase the frequency of CD4+ effector/memory T cells (Fig. 1a,c). The Otub1-TKO and wild-type (WT) mice had similar frequencies of regulatory T (Treg) cells and the Otub1-deficient Treg cells were fully functional in suppressing naive CD4+ T cells (Supplementary Fig. 2a–c). Mixed-bone marrow adoptive transfer
studies revealed that Otub1-TKO CD8+ T cells had higher frequencies of the effector/memory-like population than WT CD8+ T cells, even in the same recipient mice (Supplementary Fig. 2d,e), suggesting a cell-intrinsic role for Otub1 in maintaining CD8+ T cell homeostasis. Furthermore, Otub1-deficient naive CD8+ T cells were hyper-responsive to in vitro activation (Fig. 1d). Similar results were obtained with naive CD8+ T cells from OT-I mice, which produce CD8+ T cells with a recombinant TCR specific for the chicken ovalbumin (OVA) peptide SIINFEKL (Fig. 1e). By contrast, Otub1 deficiency had no effect on naive CD4+ T cell activation (Fig. 1d).

To examine the in vivo function of Otub1, we used a bacterial infection model employing a recombinant Listeria monocytogenes strain expressing chicken ovalbumin, LM-OVA. The Otub1-TKO mice displayed markedly enhanced immune responses against LM-OVA infection, as demonstrated by reduced liver bacterial load and increased frequencies of antigen-specific CD8+ effector T cells producing IFN-γ (Fig. 1f,g). Similar results were obtained using WT and Otub1-TKO OT-I mice producing OVA-specific CD8+ T cells (Fig. 1h). These results suggest that Otub1 maintains CD8+ T cell homeostasis and negatively regulates CD8+ T cell activation.

Otub1 regulates CD8+ T cell responses to IL-15. The γc family cytokines IL-7 and IL-15 are important for T cell homeostasis \cite{1,2}. While IL-7 regulates both CD4+ and CD8+ T cells, IL-15 is particularly important for regulating CD8+ T cells that express high levels of IL-15Rα and γc (refs. \cite{3,4}). Because Otub1 deficiency had a selective effect on CD8+ T cells (Fig. 1a), we tested whether Otub1 played a role in regulating CD8+ T cell responses to IL-15 by performing mixed-CD8+ T cell transfer using Il15ra+/+ or Il15ra−/− recipient mice (Fig. 2a). Because IL-15Rα is required for IL-15 transpresentation, T cells transferred to Il15ra−/− mice are defective in IL-15 stimulation \cite{3,4}. In the Il15ra−/− recipients, Otub1-TKO CD8+ T cells had much higher frequencies of memory-like T cells than WT CD8+ T cells (Fig. 2b,c). However, this phenotype was no longer significant.
in Il15ra+/+ recipients, suggesting a role for Otub1 in controlling CD8+ T cell responses to IL-15 (Fig. 2b,c).

We also examined the effect of Otub1 deficiency on IL-15-mediated CD8+ T cell proliferation under lymphopenic conditions. For this, we used OT-I CD8+ T cells: because the OT-I TCR does not respond to commensal antigens, OT-I T cell expansion is mediated by homeostatic cytokines, predominantly IL-7 and IL-15 (refs. 6-8). WT OT-I T cells proliferated to a similar extent in Il15ra+/+ and Il15ra−− recipient mice (Fig. 2d), consistent with the involvement of both IL-7 and IL-15 in mediating lymphopenic T cell proliferation. However, the hyperproliferation of Otub1-TKO OT-I T cells was critically dependent on IL-15, as it was largely
eliminated in the Il15ra−/− recipient mice (Fig. 2d). These results further emphasize a crucial role for Otub1 in controlling CD8+ T cell responses to the homeostatic cytokine IL-15.

**IL-15 primes CD8+ T cells for activation under the control of Otub1.** Our finding that Otub1 deficiency promoted the activation of CD8+ T cells by TCR–CD28 signals indicated that homeostatic exposure of CD8+ T cells to IL-15 might prime them for activation by antigens. In further support of this idea, the hyper-responsive phenotype of Otub1−/TKO CD8+ T cells was detected on the Il15ra+/+, but not Il15ra−/−, background (Supplementary Fig. 3a). Furthermore, in a T cell adoptive transfer experiment, Otub1−/TKO OT-I CD8+ T cells isolated from Il15ra+/+ recipients, but not Il15ra−/− recipients, displayed the hyperactivation phenotype (Fig. 2e). As an in vivo model, we performed LM-OVA infection using Il15ra+/+ or Il15ra−/− mice adoptively transferred with a mixture of WT and Otub1−/TKO naive OT-I CD8+ T cells (Supplementary Fig. 3b,c). In Il15ra+/+ recipients, Otub1−/TKO OT-I T cells displayed a much stronger response to LM-OVA infection than WT OT-I T cells, but this phenotype was not detected in the Il15ra−/− recipients (Fig. 2f and Supplementary Fig. 3d). Thus, Otub1 controls IL-15-mediated priming of CD8+ T cells for antigen-specific responses both in vitro and in vivo.

RNA sequencing revealed that Otub1−/TKO naive OT-I T cells had upregulated expression of a large number of genes under homeostatic conditions (Supplementary Fig. 3e), including signatures associated with effector/memory functions and stem cell memory T (Tscm) cells (Fig. 2g). To examine whether this gene expression pattern was dependent on IL-15 signaling, we performed quantitative PCR with reverse transcription (RT–qPCR) analysis using WT and Otub1−/TKO CD8+ T cells isolated from adoptively transferred Il15ra+/+ or Il15ra−/− recipient mice (Fig. 2h). Within the Il15ra+/+ recipient mice, Otub1−/TKO CD8+ T cells displayed upregulated expression of almost all of the genes analyzed as compared to the WT CD8+ T cells (Fig. 2h). However, within the Il15ra−/− recipient mice, WT and Otub1−/TKO CD8+ T cells no longer displayed differences in gene expression and both displayed reduced levels of gene expression as compared to CD8+ T cells derived from the Il15ra+/+ recipient mice (Fig. 2h). Together, these results suggest that, under homeostatic conditions, IL-15 primes CD8+ T cells for responding to TCR–CD28 signals and that this process is negatively regulated by Otub1.

**Otub1 also regulates NK cell maturation and activation.** NK cells also express high levels of IL-15Rβ-γ, heterodimer and rely on IL-15 for maturation and activation15. On the basis of cell-surface expression of CD11b and CD27, NK cells can be divided into four maturation stages with progressive acquisition of effector functions: stage 1 (CD11bCD27−), stage 2 (CD11b+CD27−), stage 3 (CD11b+CD27−) and stage 4 (CD11b+CD27+). IL-15 deficiency impairs generation of stage 3 and stage 4 NK cells, whereas IL-15 overexpression causes predominant accumulation of stage 4 NK cells. To study the function of Otub1 in NK cell regulation, we inductively deleted Otub1 in adult mice using a tamoxifen-inducible Cre (CreER) system (Fig. 3a,b). As expected from the Otub1−/TKO result (Fig. 1a), mice with induced knockout of Otub1 (Otub1−/iKO mice) had increased frequencies of memory-like CD8+ T cells in the spleen (Fig. 3c). Importantly, although Otub1 deletion had no effect on total NK cell numbers in the spleen, it markedly increased the frequency of stage 4 mature NK cells (CD11bCD27+) and concomitantly reduced the frequency of stage 3 NK cells (CD11b+CD27−) (Fig. 3d,e). Consistent with this, Otub1−/iKO NK cells were hyper-responsive to cytokine-stimulated activation, detected on the basis of production of granulocyte B and the chemokine CCL5 (Fig. 3f–h), which mediate NK cell effector function and recruitment of type 1 conventional dendritic cells (cDC1s), respectively.37 These results suggest that Otub1 controls the maturation and activation of NK cells, further emphasizing the role of this DUB in regulating IL-15 responses.

**Otub1 regulates the AKT axis of IL-15 receptor signaling.** Stimulation of naive CD8+ T cells with IL-15 triggered activation of the transcription factor Stat5 and the kinase AKT, as shown by their site-specific phosphorylation (Fig. 4a). Otub1 deficiency did not affect Stat5 activation but enhanced activation of AKT (Fig. 4a). AKT activation is mediated by phosphorylation at T308 and S473. Phosphorylation of AKT at T308 is crucial for activation of the protein tyrosine kinase Zap70, the adaptor protein SLP76 or TCR signaling. Otub1 deficiency did not influence phosphorylation of Zap70, SLP76 or TCR signaling (Supplementary Fig. 4a). Nevertheless, phosphorylation of AKT at T308 was also enhanced in Otub1-deficient CD8+ T cells (Supplementary Fig. 4a). Otub1 deficiency only had a weak effect on IL-2- and IL-7-stimulated phosphorylation of AKT (Supplementary Fig. 4b). Notably, the receptors of IL-2 and IL-15 have two common subunits, IL-2/IL-15Rβ and γc, although these two cytokines display different biological functions38. Our finding also suggested signaling differences between these two closely related cytokines. The role of Otub1 in regulating IL-15-stimulated AKT activation was further demonstrated using an IL-15-responsive T cell line, 15R-KIT (human KIT-225 cell line stably transfected with IL-15Rα). Otub1−/TKO knockdown in 15R-KIT T cells strongly promoted IL-15-stimulated AKT phosphorylation (Fig. 4b). Furthermore, Otub1 deficiency in NK cells also enhanced IL-15-stimulated activation of AKT, but not activation of Stat5 (Fig. 4c). Thus, Otub1 controls the AKT axis of IL-15R signaling in both CD8+ T cells and NK cells.

Because Otub1−/TKO-deficient CD8+ T cells were hyper-responsive to TCR–CD28 stimulation in vitro and antigen-specific responses in vivo (Fig. 1d–f), we examined the effect of Otub1 deletion on TCR signaling. Otub1 deficiency did not influence phosphorylation of the protein tyrosine kinase Zap70, the adaptor protein SLP76 or the MAP kinase ERK (Supplementary Fig. 4c). However, Otub1 deficiency markedly enhanced TCR–CD28-stimulated activation of AKT and phosphorylation of several AKT downstream proteins, including the transcription factors Foxo1 and Foxo3 and the mTORC1 targets S6 kinase (S6K), ribosomal S6 protein and 4E-BP1 (Fig. 4d). On the other hand, Otub1 deficiency did not affect TCR–CD28-stimulated AKT signaling in CD4+ T cells (Supplementary Fig. 4d), consistent with the finding that Otub1 controlled the activation of CD8+, but not CD4+, T cells (Fig. 1d).

To examine whether the TCR–CD28-stimulated AKT hyperactivation in Otub1−/TKO-deficient CD8+ T cells was due to IL-15 priming, we adoptively transferred WT or Otub1−/TKO naive OT-I T cells to Il15ra+/+ or Il15ra−/− recipient mice and sorted the transferred T cells for AKT activation assays (Fig. 4e). Otub1−/TKO OT-I CD8+ T cells isolated from Il15ra+/+, but not Il15ra−/−, recipient mice displayed hyperactivation of AKT (Fig. 4f), suggesting that IL-15 primes CD8+ T cells for the AKT axis of TCR–CD28 signaling under the control of Otub1. In an effort to further explore the mechanism by which Otub1 selectively regulates AKT signaling in CD8+ T cells, we found that CD8+, but not CD4+, T cells contained abundant membrane-associated Otub1 (Fig. 4g). Similarly to CD8+ T cells, NK cells also contained a high level of membrane-associated Otub1 (Fig. 4g). The membrane association of Otub1 was not affected by TCR–CD28 signaling (Fig. 4h) but was critically dependent on IL-15, as it was diminished in CD8+ T cells derived from an IL-15-α-deficient host in a T cell transfer study (Fig. 4i,j). Antibody-mediated IL-15 neutralization in WT OT-I mice also inhibited Otub1 membrane
localization in CD8+ T cells (Fig. 4k). Because AKT activation occurs in various membrane compartments16, these findings provide insight into the mechanism underlying the function of Otub1 in the regulation of AKT.

Otub1 inhibits K63 ubiquitination and PI3K binding by AKT. A key step in the activation of AKT is its recruitment to membrane compartments via interaction of its pleckstrin homology (PH) domain with the membrane lipid phosphatidylinositol-3,4,5-trisphosphate (PIP3) (ref. 34). Once in the membrane, AKT is phosphorylated at T308 and S473 by PDK1 and mTORC2, respectively. We found that IL-15 stimulated membrane translocation of AKT, which was greatly enhanced by Otub1 knockout (Fig. 5a). OTUB1 knockout had no obvious effect on the activity of AKT upstream regulators phosphatidylinositol-3-OH kinase (PI(3)K) and phosphatase and tensin homolog (PTEN) (data not shown), which catalyze the forward and reverse reactions that generate PIP3, respectively35. Interestingly, AKT was physically associated with OTUB1 in 15R-KIT cells and the association was strongly enhanced upon IL-15 stimulation (Fig. 5b). In primary OT-I CD8+ T cells, the AKT–Otub1 interaction was barely detectable at steady state but was strongly induced by IL-15 (Fig. 5c). Otub1–AKT binding was also readily detected under transfection conditions (Supplementary Fig. 4e).

Because Otub1 is a DUB, we next examined whether Otub1 regulated the ubiquitination of AKT. IL-15 stimulated ubiquitination of AKT, which was enhanced upon OTUB1 knockdown (Fig. 5d,e). Conversely, OTUB1 overexpression inhibited AKT ubiquitination, which was efficient for K63-linked, but not K48-linked, polyubiquitin chains (Fig. 5f). A previous study identified three catalytic residues of Otub1: C91, D88 and H265 (ref. 36). We found that substitution of C91 only moderately inhibited the function of OTUB1 (data not shown), but simultaneous alteration of D88 and C91 generated an OTUB1 mutant that was unable to inhibit AKT ubiquitination (Fig. 5f). WT Otub1, but not the D88A/C91S mutant, was also able to suppress AKT activation in reconstituted Otub1-deficient CD8+ T cells and OTUB1 knockdown 15R-KIT cells (Supplementary Fig. 4f,g), thus suggesting that Otub1-mediated inhibition of AKT K63 ubiquitination contributes to the negative regulation of AKT activation.

TRAF6 is known to mediate growth-factor-induced AKT ubiquitination at K8 and K14 in cancer cells16. We found that alteration
of K14 also abolished AKT ubiquitination under basal and IL-15-stimulation conditions (Fig. 5g,h). However, alteration of K8 had no effect on AKT ubiquitination (Fig. 5g,h). Consistent with this, alteration of K14, but not K8, abolished AKT phosphorylation (Fig. 5i), suggesting that ubiquitination of AKT at K14 mediates activation by IL-15. To further assess the function of AKT K63 ubiquitination, we depleted AKT K63 ubiquitination (Fig. 5k). Fusion of UbK63 to AKT K14R largely rescued its defect in IL-15-stimulated phosphorylation as well as in ubiquitination (Fig. 5k,l), suggesting that the fused UbK63 could serve as an acceptor ubiquitin for polyubiquitin chain formation and, thus, AKT activation.

AKT normally exists in a closed conformation owing to the intramolecular interaction between its N-terminal PH domain and C-terminal kinase domain38. Because ubiquitination often causes conformational changes, we surmised that ubiquitination of AKT might promote its PI3K-binding activity. While WT AKT and AKT K8R displayed strong PI3K-binding activity, the AKT K14R mutant was defective in PI3K binding (Fig. 5m). Moreover, OTUB1 strongly inhibited the PI3K-binding activity of WT AKT and AKT K8R, but it did not affect the residual PI3K-binding activity of the K14R mutant (Fig. 5m). Fusion of UbK63 to AKT(K14R), which restored its ubiquitination (Fig. 5i), completely restored PI3K-binding function (Fig. 5n). These results suggest that Otub1 deubiquitinates AKT to interfere with the PI3K binding and membrane translocation of AKT, thereby inhibiting its phosphorylation and activation.

**Otub1 regulates important gene signatures and metabolic programming in activated CD8+ T cells.** RNA sequencing analysis of in vitro-activated CD8+ T cells revealed that Otub1-deficient CD8+ T cells had 1,254 significantly upregulated and 297 significantly downregulated genes as compared to WT CD8+ T cells (Supplementary Fig. 5). The upregulated genes included those involved in activation and effector function or survival of CD8+ T cells.
Fig. 5 | Otub1 inhibits K63 ubiquitination, PIP3 binding and membrane translocation of AKT. a, Immunoblot analysis of AKT in membrane and cytosol fractions of IL-15-stimulated 15R-KIT T cells transduced with either a control shRNA or two different OTUB1 shRNAs. b, c, Co-immunoprecipitation (IP) analysis of endogenous Otub1–AKT interaction in IL-15-stimulated 15R-KIT T cells (b) and primary OT-I CD8+ T cells (c). IB, immunoblod; d, AKT ubiquitination analyses in IL-15-stimulated 15R-KIT T cells stably expressing HA–ubiquitin. e, AKT ubiquitination analysis in IL-15-stimulated OTUB1-knockdown and control 15R-KIT T cells stably expressing HA–ubiquitin. f, AKT ubiquitination analyses in HEK293T cells transiently transfected with HA-tagged WT, K63 or K48 ubiquitin in the presence (+) or absence (−) of the indicated expression vectors. The OTUB1 mutant harbors D88A/C91S substitutions. g, h, Ubiquitination analysis of WT and mutant forms of human AKT in transiently transfected HEK293T cells (g) or IL-15-stimulated 15R-KIT T cells stably expressing WT or mutant HA–AKT (h). i, Immunoblot analysis of phosphorylated and total AKT immunoprecipitated from IL-15-stimulated 15R-KIT T cells stably expressing WT or mutant human AKT. j, k, Schematic of ubiquitin K63 (UbK63)–AKT and UbK63–AKT K14R (j) and immunoblot analysis of their phosphorylated and total protein levels after immunoprecipitation from stably infected 15R-KIT T cells stimulated with IL-15 (k). l, Immunoblot analysis of ubiquitinated (top and total) AKT or UbK63–AKT proteins immunoprecipitated from transiently transfected HEK293T cells. m, Immunoblot analysis of PIP3–bound (top and total) AKT proteins isolated from transiently transfected HEK293T cells by PIP3 bead pull-down and anti-HA immunoprecipitation, respectively. n, Immunoblot analysis of AKT or UbK63–AKT proteins isolated from transiently transfected HEK293T cells by PIP3 bead pull-down (left) and anti-HA immunoprecipitation (right). Data summarize two (a,k) or three (b–i, l–n) independent experiments.

We next performed Seahorse extracellular flux analyses to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), indicators of aerobic glycolysis and oxidative phosphorylation, respectively. As compared to WT CD8+ T cells, Otub1-deficient CD8+ T cells had enhanced ECAR and maximum glycolytic capacity (stressed ECAR) under activated conditions (Fig. 6c,d). Unlike glycolysis, OCR was not significantly altered by Otub1 deficiency (Fig. 6c.f). Otub1 appeared to regulate glycolysis through controlling AKT, as a selective AKT inhibitor (AKTi) erased the ECAR differences between WT and Otub1-TKO CD8+ T cells (Fig. 6g,h). The AKT inhibitor also blocked TCR–CD28-stimulated hyperexpression of the glycolysis-regulatory genes Glut1 and Pd1, Vista and CD160 (Fig. 6a). The most striking result was the upregulated expression of a metabolic gene signature in Otub1-deficient CD8+ T cells, particularly those involved in the glycolytic pathway, such as glucose transporter 1 (Glut1, also called Slc2a1) and hexokinase 2 (Hk2) (Fig. 6a and Supplementary Fig. 5).

Immunoblot analyses confirmed the drastic upregulation of Hk2, an enzyme catalyzing the first step of the glycolytic pathway, in Otub1-TKO CD8+ T cells (Fig. 6b). These findings are intriguing, as metabolic reprogramming is a hallmark of T cell activation and is required for the function of effector T cells10-12.

T cells (Fig. 6a). The major downregulated genes included those encoding the pro-apoptotic factor Bim and immune checkpoint molecules (Pd1, Vista and CD160) (Fig. 6a). The most striking result was the upregulated expression of a metabolic gene signature in Otub1-deficient CD8+ T cells, particularly those involved in the glycolytic pathway, such as glucose transporter 1 (Glut1, also called Slc2a1) and hexokinase 2 (Hk2) (Fig. 6a and Supplementary Fig. 5). Immunoblot analyses confirmed the drastic upregulation of Hk2, an enzyme catalyzing the first step of the glycolytic pathway, in Otub1-TKO CD8+ T cells (Fig. 6b). These findings are intriguing, as metabolic reprogramming is a hallmark of T cell activation and is required for the function of effector T cells10-12.
and Hk2 and cytokine production in Otub1-TKO CD8+ T cells (Fig. 6i,j). These results suggest that Otub1 controls glycolysis induction in activated CD8+ T cells via a mechanism that involves regulation of AKT signaling.

Otub1 deficiency impairs CD8+ T cell self-tolerance. IL-15 is known to reduce the threshold of T cell activation and sensitizes CD8+ T cells for response to self-antigens.62,63 We examined the role of Otub1 in regulating CD8+ T cell self-tolerance using a well-defined mouse model, Pmel1, which produces CD8+ T cells with a transgenic TCR specific for the melanocyte self-antigen gp100 (ref. 44). Pmel1 CD8+ T cells are normally tolerant to the self-antigen gp100 when examined up to 9 months of age, 100% of the Otub1-TKO Pmel1 mice developed severe vitiligo, starting from around 3 months of age and becoming more severe over time (Fig. 7a and data not shown). While WT Pmel1 CD8+ T cells were predominantly in a naive state, a large proportion of Otub1-TKO Pmel1 CD8+ T cells were activated, displaying CD44 and CXCR3 activation markers (Fig. 7b,c). Furthermore, Otub1-TKO, but not WT, Pmel1 T cells responded to in vitro restimulation with the antigen gp100 by producing IFN-γ (Fig. 7d). These results suggest that Otub1 controls CD8+ T cell responses to microbial antigens and self-antigens in vivo.

Otub1 regulates anticancer immunity via both T cells and NK cells. Although tolerance prevents autoimmunity, it poses a major obstacle to immune responses against cancer and a general principle of cancer immunotherapy is to overcome immune tolerance.46,47 Our finding that Otub1 controls the activation of CD8+ T cells and NK cells, which are central components for cancer immunity,1,2 suggested a role for Otub1 in regulating antitumor immunity. We first tested the T cell-specific function of Otub1 by employing the Otub1-TKO mice and the murine melanoma model B16-OVA (in which B16 cells express the surrogate antigen ovalbumin). In comparison to WT mice, Otub1-TKO mice had significantly reduced tumor burden (Fig. 8a,b) coupled with increased frequencies of CD8+ effector T cells producing IFN-γ and granzyme B in both tumors and draining lymph nodes (Fig. 8c). Furthermore, Otub1-TKO CD8+ T cells expressed higher levels of Glut1 than WT CD8+ T cells in the tumor microenvironment (Fig. 8d), consistent with the role of Otub1 in regulating glycolysis (Fig. 6c,d).

To examine the therapeutic potential of targeting Otub1, we employed a mouse model of adoptive T cell therapy.47,48 We inoculated B6 mice with B16F10 melanoma cells and then treated the tumor-bearing mice by adoptive transfer of in vitro-expanded CD8+ T cells derived from WT or Otub1-TKO Pmel1 mice (Fig. 8e). Pmel1 CD8+ T cells recognize the tumor antigen gp100 expressed by B16F10 tumors. In comparison to the WT Pmel1 CD8+ T cells, Otub1-TKO Pmel1 CD8+ T cells were profoundly more effective in suppressing
tumor growth and improving survival of the B16 tumor-bearing mice (Fig. 8g).

We next employed the Otub1-iKO model, in which Otub1 is inducibly deleted in adult mice in different cell types and challenged with B16F10 tumor cells (Fig. 8h). The Otub1-iKO mice had greatly reduced tumor burden as compared to WT mice (Fig. 8i,j) associated with increased number of tumor-infiltrating CD8+ T cells and NK cells as well as CD4+ T cells and cDC1 cells (Fig. 8k). Moreover, tumor-infiltrating CD8+ T cells in the Otub1-iKO mice had a significantly higher frequency of effector cells expressing IFN-γ and granzyme B (Fig. 8l). Similar results were obtained with the MC38 colon cancer model (Supplementary Fig. 6a–c). Antibody-mediated depletion of either CD8+ T cells or NK cells impaired the potent anticancer immunity of Otub1-iKO mice, causing an increase in tumor burden to a level similar to or higher than that in WT mice (Fig. 8m,n and Supplementary Fig. 6d,e). NK cell depletion in Otub1-iKO mice drastically reduced the frequency of tumor-infiltrating cDC1 and CD4+ T cells, whereas CD8+ T cell depletion partially reduced the frequency of tumor-infiltrating cDC1 cells, but not CD4+ T cells (Fig. 8o). These results suggest that hyperactivation of CD8+ T cells and NK cells contributes to the strong anticancer immunity in Otub1-iKO mice.

To assess the role of OTUB1 in regulating antitumor immunity in human cancers, we analyzed cancer databases for potential correlation of OTUB1 expression with a T cell gene signature in tumors. Interestingly, our analysis of human skin cutaneous melanoma databases revealed a remarkable inverse correlation between OTUB1 expression levels and the abundance of the CD8+ effector T cell gene signature as well as between OTUB1 expression levels and patient survival (Supplementary Fig. 7). Collectively, these results establish OTUB1 as an important regulator of antitumor immunity and implicate Otub1 as a potential target for cancer immunotherapy.

**Discussion**

The results presented here suggest a ubiquitin-dependent mechanism that regulates IL-15R signaling and the IL-15-dependent homeostasis of CD8+ T cells and NK cells and establish the DUB Otub1 as a crucial regulator. Otub1 controls IL-15-stimulated ubiquitination and activation of AKT, a kinase that mediates the activation and metabolic reprogramming of CD8+ T cells. Despite the abundant expression of Otub1 in CD4+ T cells, Otub1 deficiency had no effect on the homeostasis of CD4+ T cells. This cell-type-specific function of Otub1 is explained by its role in regulating IL-15R signaling, which is specifically required for the homeostasis of CD8+ T cells and NK cells.

Our data suggest that homeostatic exposure of CD8+ T cells to IL-15 serves as a crucial priming step for antigen-specific CD8+ T cell activation, which is controlled by Otub1. T cell-specific deletion of Otub1 rendered CD8+ T cells hyper-responsive to bacterial infections in vivo and to activation by TCR–CD28 signals in vitro. This phenotype was due to aberrant priming of the naive CD8+ T cells by IL-15, as it was not detected in IL-15R-deficient mice. In CD8+ T cells and NK cells, Otub1 localizes to the membrane compartment. The membrane localization of Otub1 was dependent on IL-15 signaling, thus implicating Otub1 as a checkpoint of IL-15-mediated CD8+ T cell priming. Because AKT activation occurs in various membrane compartments13, these findings suggest that the membrane localization of Otub1 may facilitate its role in regulating AKT activation.

Otub1 regulates different aspects of CD8+ T cell activation and function. Otub1 deficiency sensitized CD8+ T cells for activation by both TCR–CD28 stimuli and *Listeria* infections and also promoted generation of antigen-specific effector cells. The crucial role of Otub1 in regulating CD8+ T cell responses was also revealed by the development of vitiligo in Otub1-TKO Pmel1 mice, which was caused by aberrant CD8+ T cell activation by the melanocyte self-antigen gp100. Another important function of Otub1 was to regulate the metabolic reprograming of activated CD8+ T cells, an essential mechanism for supporting proliferation and the generation and function of effector cells40. This function of Otub1 is in line with its role in AKT regulation, as AKT is a master kinase mediating the activation, metabolism and effector functions of CD8+ T cells40–42.

Inducible deletion of Otub1 in adult mice promoted tumor rejection, which was associated with increased tumor infiltration by various immune cells, including CD8+ T cells, NK cells, CD4+...
Otub1 regulates anticancer immunity. a–c, Tumor growth curve (a), day 18 tumor weight (b) and frequency of CD8^+ T cells and effector (IFN-γ^+ and granzyme B^+) CD8^+ T cells (expressed as a percentage of CD8^+ T cells) in the tumor and draining lymph nodes (dLN) (c) of WT or Otub1^−/−KO mice injected subcutaneously with 2 x 10^5 B16-OVA cells (WT, n = 6; TKO, n = 5). d, Flow cytometry analysis of Glut1 expression in tumor-infiltrating CD8^+ T cells. e–g, Schematic of experimental design (e), tumor growth curve (f) and Kaplan–Meier survival plot (g) of B6 mice that were inoculated with B16F10 melanoma cells and subsequently irradiated and injected with WT and Otub1^−/−TKO Pmel1 T cells (6 x 10^5) activated in vitro with anti-CD3 and anti-CD28 for 5 days. Control mice were inoculated with B16F10 cells without irradiation and Pmel1 T cell injection. Control, n = 4; WT Pmel1, n = 5; TKO Pmel1, n = 5. h–l, Schematic of experimental design (h), tumor growth curve (i), day 22 tumor weight (j), frequency of tumor-infiltrating immune cells (k) and frequency of tumor-infiltrating effector (IFN-γ^+ and granzyme B^+) CD8^+ T cells (expressed as a percentage of CD8^+ T cells) (l). m–o, Tumor growth curve (m), day 21 tumor weight (n) and frequency of tumor-infiltrating immune cells (o) in WT and Otub1^−/−KO (iKO) mice inoculated with B16F10 melanoma cells and, where indicated, injected with antibodies for NK cell and CD8^+ T cell depletion (anti-NK1.1 and anti-CD8a, respectively) as depicted in Supplementary Fig. 6d. Data are representative of two (a–g) or three (h–o) independent experiments each with multiple biological replicates. Summary data are shown as mean ± s.e.m. with P values determined by two-way analysis of variance (ANOVA) with Bonferroni correction (f,i,m), two-tailed Student’s t test (b–d, j–l, n–o) or log rank test (g). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; NS, not significant.
associated accession codes are available at https://doi.org/10.1038/s41590-019-0405-2.

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Otub1 transgenic mice (on the B6 genetic background and from Jackson Laboratories) to Otub1 knockout or TKO mice. The Otub1 mice were also crossed with ROSA26-CreER (Jackson Laboratories) to generate Otub1<sup>fl/fl</sup> mice. ROSA26-CreER and Otub1<sup>fl/fl</sup>, ROSA26-Cre-ER mice, which were then injected with (transperitoneal) transgenic (C57BL/6J) in corn oil for four consecutive days to induce Cre function for generation of WT and iKO mice. OT-1 and Pmel1 TCR-transgenic mice, B6.SJL (CD45.1<sup>+</sup>), C57BL/6, Rag1 knockout and Il2ra<sup>−/−</sup> knockout mice were from Jackson Laboratories. Experiments were performed with young adult (6- to 8-week-old) female and male mice except where indicated otherwise. All mice were on the B6 genetic background and maintained in a specific pathogen-free facility of the University of Texas MD Anderson Cancer Center, and all animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Cell lines. The HEK293T, B16F10 and MC38 cell lines were from ATCC and B16-OVA cells were provided by Q. Yi (Cleveland Clinic). The KIT-225 T cell line stably transfected with IL-15R (15K-RIT) was provided by S. Dubois (National Cancer Institute (NCI)/National Institutes of Health (NIH)) and cultured in RPMI 1640 medium supplemented with 10% FBS, antibiotics and human IL-2 (0.5 ng/mL).

Plasmids, antibodies and reagents. pMIG11-HA-akt was generated by inserting human AKT1 cDNA into the EcoRI and BgII sites of the retrovirus vector pMIG11 downstream of a hemagglutinin (HA) tag, and the AKT mutants (K89R, K86M and E17K) were created by site-directed mutagenesis. DNA expression vectors for Flag-tagged Otub1 and the Otub1 C91S mutant were provided by D. Durocher (Lunenfeld–Tanenbaum Research Institute) and the Flag–Otub1 C91S/D88A mutant was generated by site-directed mutagenesis. pPRC-chip-Otub1-HA and pPRC-chip-Otub1(C91S/D88A)-HA were generated by inserting human Otub1 and Otub1 C91S/D88A into the pPRC-chip-HA retroviral vector (provided by P. Martin, University of Nice Sophia Antipolis). PRK5-HA-ubiquitin WT, K63 and K48 were obtained from Addgene (plasmid numbers 17608, 17605 and 17606). Ubiquitin K63 and K48 harbor lysine-to-arginine substitutions at all lysines, except K63 and K48, respectively. pLenti-puro-HA-ubiquitin was obtained from Addgene (plasmid number 74318) and pLenti-puro-HA-Akt was generated by inserting human AKT1 cDNA into pLenti-puro-HA-ubiquitin immediately downstream of the ubiquitin cDNA. pLenti-puro-HA-ub-Akt(K14R) was created by site-directed mutagenesis. pLenti-puro-HA-Ub-K63<sup>AKT</sup>-K14R was created by replacing WT ubiquitin with K63 ubiquitin in the pLenti-puro-HA-Akt and pLenti-HA-ub-Akt(K14R) vectors. T7-AKT was generated by inserting human AKT1 cDNA into the BamH1 and Xho1 sites of the T7-RelA vector (Addgene, plasmid number 21984) to replace the RelA cDNA.

Functional-grade anti-mouse (m) CD3 (145-2C11) and anti-mCD28 (29F.1A) were from BioLegend and antibody to Glut1 (EPR3915) was from Abcam. Recombinant mouse (m) CD3 (L3T4), mCD8 (53-6.7), mCD3 (145-2C11), CD44 (IM7), mCD62L (MEL-14), mCD8 (H-2D<sup>b</sup>), mCD4 (GK1.5), mCD25 (29F.1A), mCD28 (30-F11), mCD11c (M1/70), mCD11b (M1/20), mIL-2 (JES6-5H4), mouse granzyme B (NZGB) and mIFN-γ (XM2.1) were purchased from Echelon. The neturalizing antibodies to CD8 (YTS169.4) and anti-NK1.1 (clone PK136) neutralizing antibodies (100 μg) as depicted in Supplementary Fig. 6d.

Adaptive cell therapy was performed using Pmel1 CD8<sup>+</sup> T cells recognizing the B16 melanoma antigen gp100. In brief, spleen cells were isolated from WT Pmel1 or Otub1<sup>-iKO</sup> Pmel1 mice and stimulated in vitro using plate coated anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml). The culture was provided with 10% IL-2 (10 ng/ml) on day 2 and CD8<sup>+</sup> T cells were purified from the culture on day 5 and used for adoptive transfer experiments. To generate tumor-bearing mice, WT B6 mice were injected subcutaneously with B16F10 melanoma cells. After 4 d, the tumor-bearing mice were subjected to whole-body irradiation (500 rad, He/Co irradiation) to induce lymphopenia. One day after irradiation, the mice were injected with in vitro-activated WT Pmel1 or Otub1<sup>-iKO</sup> Pmel1 CD8<sup>+</sup> T cells (6x10<sup>4</sup>). Control mice were not irradiated or injected with Pmel1 T cells. Tumor size was measured every other day for the indicated time period.

Mixed-bone marrow and mixed-T cell adoptive transfer. Bone marrow cells (2x10<sup>6</sup>) isolated from Otub1<sup>-iKO</sup> (CD45.2<sup>+</sup>) mice were mixed with bone marrow cells from WT B6.SJL (CD45.1<sup>+</sup>) mice in a 1:1 ratio and adoptively transferred into irradiated (1,000 rad) Rag1 knockout mice. After 6 weeks, the bone marrow chimeric mice were killed to allow analysis of the homeostasis of T cells derived from WT B6 (8.5 × 10<sup>6</sup>) and Otub1<sup>-KO</sup> bone marrow cells by flow cytometry on the basis of the CD45.1 and CD45.2 congenic markers.

For mixed-T cell transfers, WT (CD45.1<sup>+</sup>) and Otub1<sup>-iKO</sup> (CD45.2<sup>+</sup>) naive CD8<sup>+</sup> T cells (WT, CD45.1<sup>+</sup>; TKO, CD45.2<sup>+</sup>) were used in some experiments. In other experiments, the B6<sup>+</sup> recipient mice were injected with [125I] labeled OVA<sub>257–264</sub> peptide (0.5 μg) in the right footpad to examine the role of IL-15 in mediating lymphopoetic proliferation of CD8<sup>+</sup> T cells. Metabolic assays. OCR and ECAR were measured with an XF96 extracellular flux analyzer (Seahorse Biosciences) in an incubator incubation in a non-buffered assay medium (Seahorse Biosciences) in an incubator with [125I] labeled OVA<sub>257–264</sub> peptide (1 μg/ml) in the right footpad to examine the role of IL-15 in mediating lymphopoetic proliferation of CD8<sup>+</sup> T cells.
T cell and NK cell purification and in vitro treatments. CD8+ and CD4+ T cells were isolated from splenocytes with anti-CD8- or anti-CD4-conjugated magnetic beads (Miltenyi Biotec). NK cells were further purified by FACS sorting to obtain the CD4+CD8+2E2+ population. The naive T cells were stimulated in replicate wells of 96-well plates (2 × 10^5 cells per well) for 66 h and the culture supernatants were analyzed by ELISA (eBioscience). NK cells were isolated from splenocytes with an NK cell isolation kit (Miltenyi). Purified NK cells were cultured with IL-2 (5 μg ml^-1), IL-12 (10 ng ml^-1) and IL-18 (10 ng ml^-1) for the indicated time periods and then subjected to flow cytometry analysis of intracellular granzyme B and CCL5.

**RNA sequencing analysis.** Naive CD8+ T cells were isolated from the spleen of young (6- to 8-week-old) WT OT-I and Otvb1-TKO OT-I mice and were either immediately lysed for RNA preparation or activated for 24 h with anti-CD3 (1 μg ml^-1) and anti-CD28 (1 μg ml^-1). Total RNA was isolated with TRizol (Invitrogen) and subjected to RNA sequencing using an Illumina sequencer in the sequencing and microarray facility of the University of Texas MD Anderson Cancer Center. The raw reads were aligned to the mm10 reference genome (build mm10), using TopHat RNA-seq alignment software. The mapping rate was 70% overall across all the samples in the dataset. HTSeq-Count was used to quantify the gene expression counts from TopHat2 alignment files. Differential expression analysis was performed on the count data using R package DESeq2. P values obtained from multiple binomial tests were adjusted using the false discovery rate (Benjamini–Hochberg). Significant genes are defined by a P value cutoff of 0.05 and fold change of at least two. RNA sequencing data were analyzed by Genesis (http://genome.tugraz.at/) and multipplot (https://genepattern.broadinstitute.org/gp/pages/login.jsf). RNA sequencing data were deposited to the Gene Expression Omnibus with the accession code GSE126777.

**Quantitative PCR.** RNA was extracted with TRizol reagent from isolated WT OT-I or Otvb1-TKO OT-I CD8+ T cells. The RNA samples were subjected to quantitative PCR analyses using SYBR reagent (Bio-Rad). The expression of individual genes was calculated by a standard-curve method and was normalized to the expression of Actrl. Gene-specific primer sets used in this study (all for mouse genes) are listed in Supplementary Table 1.

**Retroviral and lentiviral infections.** Retroviral particles were prepared using the indicated expression vectors, which are based on pMIGR1-GFP or pPRIChp-α-hERG. HEK293T cells were transduced (using the calcium method) with pGIPZ lentiviral vectors encoding OTUB1-specific shRNAs or a non-silencing control shRNA along with the packaging vectors pSAX2 and pMD2. T cells (15R-KIT) were infected with the recombinant retroviruses or lentiviruses. For primary T cell infection, naive OT-I CD8+ T cells were stimulated in 12-well plates for 24 h with plate-bound anti-CD3 (1 μg ml^-1) plus anti-CD28 (1 μg ml^-1) in the presence of 10 ng ml^-1 IL-15 and 5 ng ml^-1 IL-2 and then infected twice (at 48 h and 72 h) with retroviruses. Twenty-four hours after the second retroviral transduction, the infected T cells were starved in low-serum (0.5% FBS) medium overnight and then stimulated with IL-15 (60 ng ml^-1) for signaling assays.

**Immunoblot, co-immunoprecipitation and ubiquitination assays.** For immunoblot analysis of protein phosphorylation, naive CD4+ and CD8+ T cells or cells from the 15R-KIT T cell line were stimulated with IL-15 (60 ng ml^-1), IL-2 (60 ng ml^-1) or IL-7 (60 ng ml^-1) for the indicated time periods and lysed in a kinase cell lysis buffer supplemented with phosphatase inhibitors. T cell stimulation with agonistic antibodies to TCR and CD28 was performed using a crosslinking method. In brief, the cells were incubated on ice with anti-CD3 (2 μg ml^-1) and anti-CD28 (2 μg ml^-1), followed by crosslinking with goat anti-hamster immunoglobulin (25 μg ml^-1) for different time periods at 37 °C and then immediately forced as described above for immunoblot assays.

**Membrane protein detection.** Membrane and cytosol protein fractions were isolated from CD4+ and CD8+ T cells or NK cells with the Mem-Per Plus Kit (Thermo Fisher) and subjected to immunoblot assays. To test the role of IL-15 in mediating Otvb1 membrane localization, OT-I mice injected intraperitoneally with a neutralizing antibody to mouse IL-15 (AIO3; 200 μg per mouse) daily for 3 days and CD8+ T cells were isolated on day 4 for preparation of membrane and cytosol protein fractions. In some experiments, a T cell adoptive transfer approach was used. In brief, OT-I CD8+ T cells were labeled with CFSE and adoptively transferred into Il15ra−/− or Il15ra+− recipient mice. After 7 days, the OT-I CD8+ T cells were isolated from recipient mice for membrane and cytosol protein preparation.

**Immune signature and survival analysis of human cancer.** To correlate the expression level of OTUB1 with the level of CD8+ effector T cells in human cancer, we collected ten well-defined CD8+ T cell-associated genes to form the immune signature. We downloaded skin cutaneous melanoma tumor samples (n = 458), including clinical and mRNA expression information, from http://www.oncolnc.org/ and submitted the compiled dataset to GenePattern (https://genepattern.broadinstitute.org/gp/pages/login.jsf) to perform unsupervised hierarchical-clustering analysis. Survival data from different clusters were used for Kaplan–Meier estimation in GraphPad Prism software.

**Statistical analysis.** For tumor clinical scores, differences between groups were evaluated by two-way ANOVA with Bonferroni correction. For survival, differences between groups were evaluated by log-rank test. Other statistical analyses were performed by two-tailed unpaired t test using GraphPad Prism software. P values less than 0.05 were considered significant and the level of significance is indicated as ***P < 0.001, **P < 0.01, *P < 0.05, **P < 0.01.***

In our animal studies, three to four mice were required for each group on the basis of our calculation to achieve a 2.3-fold change (effect size) in a two-tailed t test with 90% power and a significance level of 5%. All statistical tests were justified as appropriate and the data met the assumptions of the tests. The variance was similar between the groups being statistically compared.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** RNA sequencing datasets were deposited to Gene Expression Omnibus with the accession code GSE126777. Other datasets generated during the current study are available from the corresponding author upon reasonable request. The human skin cutaneous melanoma datasets reported by other studies were downloaded from http://www.oncolnc.org/.

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|-----------------|--------------------------------------------------|--------------------------------|--------------------------------|--------------------------------|

| Data analysis   | Statistical analysis: GraphPad Prism(Ver 8) | Flow cytometric analysis: Flowjo( ver. 9.7.7 and Ver 10 for Mac) | RNA seq data analysis: HTseq-Count , R package Tophat2 RNASeq alignment software, Multiploid in Genepattern from Broad institute, | Genesis (Version 1.8.1) |
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| Sample size | Sample sizes were chosen according to the basis of previous publications in the immunology field, without prior power analysis. We usually used more than 4-5 mice per group to ensure the statistically significant difference could be obtained from unpaired two-tailed Student's t-test, ANOVA analysis with Bonferroni's post-test or Log-rank test. We described the exact numbers of animals/samples for each experiment in the figure legends. |
| Data exclusions | No data were excluded from the analysis |
| Replication | The experimental findings were reliably reproduced. The replication numbers were described in the corresponding figure legends |
| Randomization | age- and sex-matched mice were assigned randomly to experimental and control groups |
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|---|---|
| n/a | n/a |
| Involved in the study | Involved in the study |
| ■ Antibodies | ■ Chip-seq |
| ■ Eukaryotic cell lines | ■ Flow cytometry |
| ■ Palaeontology | ■ MRI-based neuroimaging |
| ■ Animals and other organisms | ■ Clinical data |
| ■ Human research participants | |
| ■ Clinical data | |

Antibodies

Antibodies used

- Functional grade anti-mouse (m) CD3e (145-2C11) and anti-mCD28 (37.51) antibodies were from eBioscience. Gcst anti-hamster IgG (H+L) was from Southern BioTech. Mouse IL-15 monoclonal antibody (A10.3) was used for in vivo IL-15 neutralization was from eBioscience. Antibodies for AKT1 (B-1), ERK1/2 (K-23), Ubiquitin (P4D1), SLF76 (H-300), Zap70 (T5.72), P65a (B-9) and PTEN (A2B1) were from Santa Cruz Biotechnology. Anti-AKT (40D4; used for IP) was from Cell Signaling, and anti-Oub1 (EPR13028(B)) was from Abcam. Anti-Aktin (C-4), and horseradish peroxidase–conjugated anti-Flag (M2) were from Sigma-Aldrich. Antibodies for phospho-AKT1 Ser473 (D9E), phospho-AKT1 Thr308 (C31E5E), phospho-FoxO1 Thr24/FoxO3a Thr32, phospho-S6K1 Thr421/Ser424, phospho-S6 Ser235/236 (D57.2E), phospho-Stat5 Tyr694 (C11E5), phospho-SLP76 Ser376, phospho-Zap70 Tyr329/Syk Tyr352, S6K1 (9707), S6 (S235), FOXO1 (C29H4), FoxO3a (75D8), HX2 (C64G5), α-Tubulin, IgG1/2b (111A9), and Stat5 were from Cell Signaling Technology. Horseradish peroxidase–conjugated anti-hemagglutinin (HA-7) was from Roche. The anti-CD8 (YTS169.4) and anti-NK1.1 (PK136) neutralizing antibodies were purchased from BioXCell. Dilution: primary antibody 1:1000 2000. secondary antibody 1:2500.

- Fluorescence-labeled antibodies for mCD4 (13TA4), mCD8 (53-6.7), mCD3 (145-2C11), CD44 (M7), mCD61 (ME-14), mTCRα (H57-597), mCD45.1 (A20), mCD45.2 (104), mCXCR3 (CXCR3-173), mFas (F11-65), mCD45.60-F11), mNK1.1 (PK136), mCD11c (N418), mMHC (M5/114-152), mCD64/54 (57.1), mCD11b (M1/70), mIL-2 (JES6SH4), mTNF-α (MP6-XT22) mGranzyme B (N68Z) and mIFNγ (XMG1.2) were purchased from eBioscience. mCD24 (M1/69) and mCD11c (M290) were from BD and mCC52 (3E9/CC5) was ordered from Biolegend. Glut1 (EPR3915) was from abcam. (from methods section). Dilution for FACS 1:200

Validation

All commercial antibodies have been validated by manufacturer's websites.
Eukaryotic cell lines

Cell line source(s) | KIT225-11.15Ra T cell line was provided by Dr. Sigrid Dubois (NCI/NIH). HEK293T, B16F10 and MC38 were purchased from American Type Culture Collection. B16-OVA cells were obtained from Dr. Qing Yi [Cleveland Clinic].

Authentication | None of the cell line have been authenticated.

Mycoplasma contamination | All cell lines were tested for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register) | No commonly misidentified lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines are recommended for reporting animal research

Laboratory animals | All mice were in B6 genetic background and maintained in a specific pathogen-free facility of The University of Texas MD Anderson Cancer Center, and all animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

The Otub1-flox mice (in B6 genetic background) were generated using embryo obtained from The European Conditional Mouse Mutagenesis Program (EUCOMM, strain Otub1tm1lal[EUCOMM]+Hmgu). Otub1-flox mice were crossed with CDA4Cre transgenic mice (both in B6 genetic background and from Jackson laboratories) to produce age-matched Otub1+/+CD4Cre (named WT) and Otub1+/+CreER mice. The Otub1-flox mice were also crossed with Rosa26-CreER (Jackson Laboratories) to generate Otub1+/+CreER and Otub1f/CreER ER mice, which were then injected i.p. with tamoxifen (2 mg per mouse) in corn oil daily for four consecutive days to induce Cre function for generation of WT and induced Otub1 KO (IKO) mice. OT-I and Pmel1 TCR-transgenic mice, B6.SJL (CD45.1+), C57Bl/6, Rag1-KO, and II15ra-KO mice were from Jackson Laboratory. Experiments were performed with young adult (6-8 weeks) female and male mice except where indicated otherwise.

Wild animals | This study did not involve the use of wild animals.

Field-collected samples | This study did not involve the use of wild animals.

Ethics oversight | This study did not involve the use of field-collected samples.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☑ All plots are contour plots with outliers or pseudocolor plots.
☑ A numerical value for number of cells or percentage [with statistics] is provided.

Methodology

Sample preparation | Spleen, peripheral draining lymph nodes, and tumor sample were gently ground under nylon mesh using the flat end of syringes. Red blood cells were removed by ACK lysing buffer, following by washing cell with isolation buffer. Cell were then filtered, pelleted and staining for FACS or sorting.

Cells were blocked with Fc blocker (CD16/32), and stained for specific surface markers. For intracellular staining, cells were fixed and permeabilized and stained for intracellular cytokines by fixation/permeabilization kit (BD bioscience).

Instrument | Flow cytometry data were collected by FACSFortessa and FACSARia.

Software | Flow cytometry data were analyzed by FlowJo software (TreeStar, Ashland, OR).

Cell population abundance | The purities of the sorted T cells were more than 99%.

Gating strategy | For immune cells, first we gated the lymphocytes based on the FSC-A and SSC-A. Singlets were gated according to the pattern of FSC-H vs FSC-A. The specific cell population was gated on the indicated surface markers as described in the manuscript.

☑ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.