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Programmed cell death 1 (PD-1) regulates the effector function of CD8 T cells via PD-L1 expressed on target keratinocytes

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Abstract

Programmed cell death 1 (PD-1) is an inhibitory molecule expressed by activated T cells. Its ligands (PD-L1 and -L2; PD-Ls) are expressed not only by a variety of leukocytes but also by stromal cells. To assess the role of PD-1 in CD8 T cell-mediated diseases, we used PD-1-knockout (KO) OVA-specific T cell-receptor transgenic (Tg) CD8 T cells (OT-I cells) in a murine model of mucocutaneous graft-versus-host disease (GVHD). We found that mice expressing OVA on epidermal keratinocytes (K14-mOVA mice) developed markedly enhanced GVHD-like disease after transfer of PD-1-KO OT-I cells as compared to those mice transferred with wild-type OT-I cells. In addition, K14-mOVA×OT-I double Tg (DTg) mice do not develop GVHD-like disease after adoptive transfer of OT-I cells, while transfer of PD-1-KO OT-I cells caused GVHD-like disease in a Fas/Fas-L independent manner. These results suggest that PD-1/PD-Ls-interactions have stronger inhibitory effects on pathogenic CD8 T cells than does Fas/Fas-L-interactions. Keratinocytes from K14-mOVA mice with GVHD-like skin lesions express PD-L1, while those from mice without the disease do not. These findings reflect the fact that primary keratinocytes express PD-L1 when stimulated by interferon- γ *in vitro*. When co-cultured with K14-mOVA keratinocytes for 2 days, PD-1-KO OT-I cells exhibited enhanced proliferation and activation compared to wild-type OT-I cells. In addition, knockdown of 50% PD-L1 expression on the keratinocytes with transfection of PD-L1-siRNA enhanced OT-I cell proliferation. In aggregate, our data strongly suggest that PD-L1, expressed on activated target keratinocytes presenting autoantigens, regulates autoaggressive CD8 T cells, and inhibits the development of mucocutaneous autoimmune diseases.

Keywords

programmed cell death 1; programmed cell death ligand 1; Fas; keratinocyte; CD8 T cell; graft-versus-host-disease

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Conflict of interest

The authors declare no financial conflict of interest.

1. Introduction

Programmed cell death 1 (PD-1) expressed on T cells is an immunoreceptor that negatively regulates antigen receptor signaling upon interacting with either of two ligands, PD-L1 and PD-L2 (PD-Ls) [1]. While PD-L1 has a wide distribution on leukocytes and stromal cells including tumor cells [2], PD-L2 is predominantly expressed on dendritic cells [3]. Upon engagement with PD-L1 or PD-L2, PD-1 on T cells inhibits TCR/CD3 signaling and CD28 signaling via phosphatase SHP2 (and possibly SHP1 as well) that dephosphorylates phosphatidylinositol-3-kinase and ZAP70/CD3 ζ [4-6]. Clinically, blockade of PD-1-PD-L1 has recently been founded to be an effective treatment for cancer by enhancing cancer immunity [7, 8]. On the other hand, PD-1 deficiency leads to lupus-like autoimmune disease with glomerulonephritis and arthritis or fatal dilated cardiomyopathy in mice [9, 10]. It has been also shown that rodent models of autoimmune disease are modulated via the PD-1-PD-Ls system. Blockade of PD-1-PD-Ls interaction with anti-PD-1/PD-L1 blocking antibodies or using PD-1/PD-L1-deficient rodents accelerates disease in prediabetic non-obese diabetic (NOD) mice, a murine model of type I diabetes [11, 12], experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis [13], MRL/MpJ-Fas (lpr) mice and (New Zealand Black \times New Zealand White)F1 mice, murine models of lupus [14, 15], and collagen-induced arthritis, a murine model of rheumatoid arthritis [16]. In aggregate, PD-1/PD-Ls system seems to regulate autoaggressive T cells during the breakdown of peripheral tolerance and development of autoimmunity.

To dissect the specific roles of autoaggressive CD8 T cells against epidermal keratinocytes in mucocutaneous diseases, we generated a transgenic mouse that expresses chicken ovalbumin (OVA) in skin and mucosal epithelia under the control of the keratin 14 promoter (K14-mOVA mouse). After adoptive transfer of transgenic T cells, OT-I cells, that express a TCR for the CD8-epitope of OVA (SIINFEKL), recipient K14-mOVA mice develop erosive skin and mucosal lesions as well as weight loss [17, 18]. Their bimodal weight loss curve may be caused by difficulty in chewing and swallowing because of mucosal lesions. In the late phase of this disease, the mice are partially saved by ingesting a gel meal. Mucocutaneous lesions in these mice show histological features characterized by liquefaction degeneration of the basal epidermal layer. This inflammatory reaction is reminiscent of human diseases including acute graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation, drug eruptions such as erythema multiforme, lupus erythematosus, dermatomyositis and lichen planus, and is called a lichenoid tissue reaction/interface dermatitis [19]. In addition to the K14-mOVA mice described above, we also generated double transgenic (DTg) mice, which are K14-mOVA mice crossed with OT-I mice. DTg mice are consistently resistant to developing GVHD-like disease after adoptive transfer of OT-I cells [20]. In a previous report, we assumed that one of the mechanisms that peripherally inhibits transferred OT-I cells might be Fas-FasL killing of transferred syngeneic OT-I cells by double negative (DN) OT-I cells (CD3⁺B220⁻CD4⁻CD8⁻V α 2⁺V β 5⁺ cells) that are increased in the DTg mice.

Here, we report that PD-1 expression on autoaggressive CD8 T cells is critical in inhibiting the development of mucocutaneous GVHD-like disease. In addition, PD-L1 expressed on

target keratinocytes regulates the antigen-specific CD8 T cells that are involved in the inflammation.

2. Results

2.1. Adoptive transfer of PD-1-knockout (KO) OT-I cells causes markedly enhanced GVHD-like disease in K14-mOVA mice and causes *de novo* GVHD-like disease in K14-mOVA/OT-I DTg mice when compared to mice adoptively transferred with wild-type OT-I cells or Fas-KO OT-I cells

K14-mOVA mice develop GVHD-like disease, weight loss and erosive skin and mucosal lesions characterized by interface dermatitis, when adoptively transferred with more than 5×10^5 OT-I cells, and 10 – 20% of them die within 2 weeks with severe weight loss. To determine whether PD-1 and Fas expressed on effector CD8 T cells have inhibitory roles in the disease, we transferred 1×10^6 wild-type OT-I cells, PD-1-KO or Fas-KO OT-I cells into K14-mOVA mice. The mice transferred with PD-1-KO OT-I cells rapidly lost weight, shivered severely and suddenly died within 4 days after the transfer without any clinical skin or mucosal lesions or pathology in internal organs (brain, heart, lung, liver and kidney), while those transferred with Fas-KO OT-I cells followed the same GVHD-like disease course as those transferred with wild-type OT-I cells (Fig. 1A). Control B6 mice do not develop GVHD-like disease after the transfer of wild-type OT-I cells. As shown in Table 1, serum levels of proinflammatory cytokines in the mice that were transferred with PD-1-KO OT-I cells were markedly elevated 3 days after transfer (just before sudden death) compared to cytokines in mice transferred with wild-type or Fas-KO OT-I cells.

We next titrated the number of transferred OT-I cells to 5×10^4 , which is far less than is required to cause GVHD-like disease in K14-mOVA mice. Only mice transferred with reduced numbers of PD-1-KO OT-I cells lost weight, and 4 of 5 mice died (Fig. 1B). The mouse that survived 14 days after the transfer of 5×10^4 PD-1-KO OT-I cells developed severe skin and mucosal lesions with erosions and crusts, characterized histologically by liquefaction degeneration of the basal epidermal cell layer, while all mice transferred with 5×10^4 wild-type or Fas-KO OT-I cells exhibited no skin or mucosal lesions (Fig. 1C and 1D).

To determine whether transferred PD-1-KO OT-I cells are activated to a greater extent than wild-type OT-I cells in K14-mOVA mice, skin-draining lymph node (SDLN) cells were analyzed by flow cytometry 7 days after the adoptive transfer of 5×10^4 wild-type or PD-1-KO OT-I cells, both expressing green fluorescence protein (GFP). There were greater numbers of PD-1-KO OT-I cells in SDLNs compared with wild-type cells (Fig. 1E). Both groups adoptively transferred with OT-I cells expressed the specific TCR ($V\alpha 2$ and $V\beta 5$), CD44 and CD25, and down-regulated expression of CD62L on their surface, and wild-type OT-I cells also expressed PD-1 (Fig. 1F). Expression of $V\alpha 2$, $V\beta 5$ and CD44 was higher and of CD62L was lower on GFP⁺OT-I cells in SDLNs of mice transferred with PD-1-KO OT-I cells compared to those transferred with wild-type OT-I cells (Fig. 1G). Both types of naïve OT-I cells express high $V\alpha 2$, $V\beta 5$, CD62L, and low CD44, CD25 and CD69 before transfer (Suppl. Fig. 1). These results demonstrate that PD-1KO OT-I cells were more

numerous and activated to a greater extent than wild-type OT-I cells in SDLNs of K14-mOVA mice.

Consistent with our prior studies [20], when DTg mice were adoptively transferred with 1×10^6 OT-I cells they did not develop GVHD-like disease. On the other hand, DTg mice that were adoptively transferred with 1×10^6 PD-1-KO OT-I cells developed severe disease with marked weight loss and skin/mucosal lesions and many died (Fig. 2A, B, C). Although we showed that double negative T cells ($CD3^+CD4^-CD8^-V\alpha 2^+V\beta 5^+$; DN T cells) found in increased numbers in LNs and spleens of DTg mice might have inhibitory effects on transferred OT-I cells via the Fas-FasL system, DTg mice that received 1×10^6 Fas-KO OT-I cells did not develop GVHD-like disease clinically (Fig. 2A, B, C). When SDLN cells were analyzed by flow cytometry 7 days after the adoptive transfer of 1×10^6 wild-type or PD-1-KO GFP⁺OT-I cells, we found that total cell numbers of PD-1-KO OT-I cells occupied larger populations of SDLNs than wild-type OT-I cells (Fig. 2E), and that PD-1KO OT-I cells transferred into DTg mice were more activated than wild-type OT-I cells (Fig 2D, E).

In aggregate, our data strongly suggest that the expression of PD-1 on effector CD8 T cells inhibits the development and progression of the CD8 T cell-mediated mucocutaneous autoimmune disease.

2.2. SDLN cells and epidermal cells from K14-mOVA mice with GVHD-like disease express the ligands of PD-1

To determine which cells express the ligands of PD-1 (PD-L1 and PD-L2; PD-Ls), SDLN cells and ear epidermal cells were collected from B6 mice and K14-mOVA mice after transfer of 1×10^6 OT-I cells and analyzed by flow cytometry. PD-L1 was expressed on CD4 T cells and dendritic cells (DCs) in SDLNs not only from K14-mOVA mice but also from B6 mice 5 days after transfer of OT-I cells (Fig 3A). The median fluorescence intensities (MFIs) of PD-L1 were significantly higher in SDLN cells from K14-mOVA mice than those from B6 mice (Fig 3C). While PD-L2 were expressed only on DCs but not on OT-I cells and CD4 T cells in SDLNs from B6 mice after OT-I transfer, these three cell types expressed PD-L2 in SDLNs from K14-mOVA mice (Fig 3A, D). Thus, PD-Ls were expressed on a wide-range immune cells.

We also analyzed epidermal cells by flow cytometry. PD-L1 were expressed on $\gamma\delta$ T cells and Langerhans cells (LCs) in the epidermis not only from K14-mOVA mice but also from B6 mice 5 days after transfer of OT-I cells (Fig 3B) whereas the MFIs were significantly higher in epidermal cells from K14-mOVA mice with GVHD-like skin lesions than those from B6 mice (Fig 3E). Interestingly, keratinocytes (KCs) of K14-mOVA mice expressed PD-L1, while those of B6 mice did not (Fig 3B, E). KCs of naïve B6 and K14-mOVA mice never express PD-L1 or PD-L2 (Suppl. Fig. 2). These results show that target KCs express PD-L1 only when activated CD8 T cells infiltrate to skin.

In addition, $\gamma\delta$ T cells and, to a lesser extent, LCs also expressed PD-L2 not only in K14-mOVA mice with skin lesions but also in B6 mice (Fig 3B, F). All $\gamma\delta$ T cells expressed PD-L2, while small populations of LC expressed PD-L2 in K14-mOVA mice transferred with

OT-I cells (Fig 3B). These results indicate that $\gamma\delta$ T cells may inhibit autoaggressive CD8 OT-I cells expressing PD-1.

2.3. Keratinocytes expressing OVA modulate the proliferation and the activation of OT-I cells

In our previous studies [21], epidermal cells devoid of LCs from K14-mOVA mice could induce naïve OT-I cells to proliferate. Although LCs and $\gamma\delta$ T cells of K14-mOVA mice with GVHD-like disease upregulated PD-Ls (Fig 3B, F), LC (CD11c⁺ cell)-depleted, $\gamma\delta$ T cell (CD3⁺ cell)-depleted or all hematopoietic cell (CD45⁺ cell)-depleted of epidermal cells from K14-mOVA mice had no significant effect on GFP⁺OT-I cell proliferation *in vitro* (Fig. 4A). We suggest that the effects of LCs and $\gamma\delta$ T cells on OT-I cell proliferation *in vitro* are minor, at best. When PD-1-KO OT-I cells were assessed in co culture experiments with K14-mOVA KCs for 3 days, they proliferated and were activated to a greater extent than wild-type OT-I cells under the stimulation of K14-mOVA KCs (Fig. 4B, C, D). Wild-type OT-I cells expressed PD-1 only when co-cultured with K14-mOVA KCs (Fig. 4E). We also observed that, in culture, PD-1-KO OT-I cells produced greater amounts of the cytotoxic molecules, IFN- γ and granzyme B, than do wild-type OT-I cells (Fig. 4E). These results indicate that K14-mOVA KCs stimulate not only TCRs but also PD-1 expressed on OT-I cells, and that PD-1 on activated OT-I cells modulates proliferation and activation of OT-I cells.

KCs upregulated PD-L1 expression when stimulated with interferon (IFN)- γ , a cytokine produced by activated OT-I cells (Fig. 5A). The viability of these KCs, when transfected with 160 μ M control- or PD-L1-specific-siRNA was reduced by 50% when compared to untreated KCs. Transfection of PD-L1-siRNA inhibited 50 % of expression of PD-L1 on keratinocytes compared with that of control-siRNA (Fig. 5A). To determine whether PD-L1 expressed on KCs inhibits the proliferation of OT-I cells, we attempted to knock down PD-L1 expressed on K14-mOVA KCs that were co-cultured with GFP⁺OT-I cells. OT-I cells co-cultured with PD-L1-siRNA-transfected K14-mOVA KCs exhibited greater proliferation than those transfected with control-siRNA-transfected KCs (Fig. 5B). In repeated experiments with PD-L1-siRNA and experiments using another PD-L1-siRNA with a different sequence, we found that transfection PD-L1-siRNA enhanced proliferation of OT-I cells by 10 – 28 %.

In aggregate, these *in vitro* results demonstrated that PD-L1 expressed on KCs presenting autoantigens inhibit the proliferation of the autoantigen-specific CD8 T cells.

3. Discussion

PD-1 expressed on pathogenic CD8 T cells plays an important role in regulating GVHD-like mucocutaneous lesions in our murine models of autoaggressive CD8 T cell-mediated disease. In the present study we show that K14-mOVA mice expressing membrane-bound OVA on epidermal keratinocytes developed GVHD-like disease after adoptive transfer of small numbers of PD-1-KO OT-I cells. It has previously been demonstrated that transgenic mice expressing membrane-bound OVA exclusively on cardiac myocytes (cMy-mOVA mice,) transferred with PD-1-KO OT-I cells develop more severe myocarditis by

immunization with OVA in *Freund's* Complete Adjuvant than those transferred with wild-type OT-I cells [22]. Similar to what has been previously reported in cMy-mOVA mice, PD-1-KO OT-I cells proliferated to a greater extent than wild-type OT-I cells in K14-mOVA mice. The mice quickly died after transfer of 1×10^6 PD-1-KO OT-I cells, and had either a high mortality (80%) or severe disease with ulcerative skin and mucosal lesions even after transfer with as few as 5×10^4 PD-1-KO OT-I cells. This finding may rely not only on the vigorous proliferation of PD-1-KO OT-I cells but also on their ability to kill SIINFEKL-presenting cells [22].

We also have observed that a few mice in groups of wild-type OT-I cell-transferred K14-mOVA mice, not only PD-1KO OT-I cell-transferred mice, suddenly died with severe shivering early (4-6 days) after cell transfer. This sudden death is probably due to a cytokine storm. We have never detected severe skin or mucosal lesions or inflammatory pathology in internal organs. As shown in Table 1, serum levels of some proinflammatory cytokines in K14-mOVA mice transferred with PD-1-KO OT-I cells were elevated compared to mice transferred with wild-type or Fas-KO OT-I cells. Because PD-1-KO OT-I cells were increased in SDLNs of K14-mOVA mice to a greater extent than were wild-type or Fas-KO OT-I cells, the increased OT-I cells in LNs are probably the source of the cytokines. We also observed that OT-I mice develop the same condition (sudden death following severe shivering) within 24 hours after an intravenous injection of SIINFEKL peptides, and that IFN γ -, TNF α - or IL-6-deficiency blocked the death (unpublished data). When transferred with lower numbers of PD-1KO OT-I cells, the K14-mOVA mice survived through an early phase of weight loss and developed severe skin and mucosal lesions in the later phase (7 – 14 days after the transfer) of GVHD-like disease.

In our mucocutaneous GVHD model, leukocytes in SDLNs upregulated both PD-L1 and PD-L2 as expected. Interestingly, KCs from K14-mOVA mice upregulated expression of PD-L1 but not PD-L2 after transfer of OT-I cells. It has been shown that KCs upregulated mRNA of PD-L1 after IFN γ -stimulation [2]. Likewise, we have shown here that PD-L1 appears on the membrane of K14-mOVA KCs when stimulated with IFN γ . Although we have already reported that OT-I cells can proliferate when co-cultured with K14-mOVA KCs devoid of any co-stimulating molecules like CD80/86 [21], PD-1-KO OT-I cells proliferated and were activated to a greater extent than wild-type OT-I cells. This finding indicates that the proliferation and activation of antigen-specific CD8 T cells may be inhibited via PD-L1 on antigen-presenting KCs. To exclude inhibitory effects of PD-Ls expressed on OT-I cells themselves, transfection of siRNA for PD-L1 was used to knockdown expression of PD-L1 on KCs. OT-I cells proliferated to a greater extent when co-cultured with PD-L1-knockdown K14-mOVA KCs than with control KCs. The enhancement of OT-I proliferation induced by transfection of PD-L1-siRNA on antigen-presenting KCs was, however, not dramatic (10 – 28%), and this may be because only 50 % of PD-L1 was knocked down. In aggregate, PD-L1, expressed on activated target keratinocytes presenting autoantigens, regulates autoaggressive CD8 T cells, and inhibits the development of autoimmune mucocutaneous disease with interface dermatitis. Interestingly, in human mucosal samples from patients with lichen planus, a mucocutaneous disease with interface dermatitis, infiltrating inflammatory cells expressed PD-1 and PD-Ls, and KCs

expressed PD-L1 [23]. In that article, it was shown that human allogeneic T-cell proliferative responses and IFN- γ production induced by IFN- γ -treated KCs were augmented preferentially by anti-PD-L1 blocking antibodies. K14-PD-L1-Tg mice were also shown to be resistant to developing contact hypersensitivity [24].

In addition to cutaneous diseases, PD-L1 expression on parenchymal cells is critical for suppression of acute GVHD in irradiated B6 mice after transfer of bone marrow (BM) cells from BALB/c mice, another murine model of GVHD. Chimeras that lack PD-L1 on parenchymal cells but not on hematopoietic cells developed GVHD to a greater extent than other chimeras lacking PD-L1 only on hematopoietic cells after transfer of BM cells from BALB/c mice [25]. While NOD severe combined immunodeficiency (SCID) mice developed diabetes after adoptive transfer of splenocytes from NOD mice, early onset of diabetes was observed in PD-L1/L2-KO NOD SCID mice [26]. Another group also showed that PD-L1-deficiency in rat-insulin promoter (RIP)-B7.1 Tg mice accelerated to the development diabetes after immunization with preproinsulin-encoding vectors [27]. In a murine model of autoimmune myocarditis that develops in cMy-mOVA mice, PD-L1/L2-KO cMy-OVA Tg mice transferred with OT-I cells developed more severe myocarditis after immunization with OVA than do wild-type cMy-OVA Tg mice [28]. In patients with malignant tumors, it has been suggested that PD-L1 expressed on tumor cells correlated with the number of infiltrating inflammatory cell around the tumors [29-32]. These reports indicate that PD-Ls expressed on stromal cells might be critical in inhibiting effector T cells more so than those on hematopoietic cells. Our results also suggest that target cells expressing PD-L1 protect themselves from autoantigen-specific CD8 T cells in autoimmune mucocutaneous disease.

When we utilized K14-mOVA \times OT-I double Tg (DTg) mice that never develop GVHD-like disease after adoptive transfer of OT-I cells, transfer of PD-1-KO OT-I cells caused GVHD-like disease. Although DN OT-I cells that were found in increased numbers in DTg mice kill syngeneic OT-I cells via Fas-FasL interactions *in vitro* [20], transfer of Fas-KO OT-I cells could not cause the disease in DTg mice as well as wild-type OT-I cells. These results suggest that PD-1/PD-Ls-interactions have stronger inhibitory effects on pathogenic CD8 T cells than do the Fas/Fas-L-interactions *in vivo*. Although activated DN OT-I cells expressed PD-L1, PD-1KO-OT-I cells were killed by DN OT-I cells after SIINFEKL-stimulation to the same extent as wild-type OT-I cells *in vitro* (Suppl. Fig. 3). In addition, treatment with PD-L1-IgG-Fc protein that stimulates PD-1 (kindly provided by Dr. G. Freeman, Harvard School of Medicine, Boston, Massachusetts) [33] did not inhibit GVHD-like disease in our model (Suppl. Fig. 4). All of these findings thus strongly suggest that expression of PD-L1 on antigen-presenting cells may be more important than that on surrounding leukocytes.

Though the pathway of PD-1-mediated programmed cell death (PCD) is still unknown, previous reports in cancer or infection showed that PD-1-expression on T cells induced PCD of the T cells [34-37]. Because it has been reported that CD3/CD8-mediated signaling induces PCD on OT-I cells more so than does TCR-mediated signaling [38], we stimulated wild-type, PD-1KO or Fas-KO OT-I cells with anti-CD3/CD8 antibodies to address whether PD-1- or Fas-deficiency inhibits PCD of OT-I cells. Stimulation by anti-CD3/CD8 antibodies accelerated PCD and reduced live cells of all types of OT-I cells (Suppl. Fig. 5).

PD-1- and Fas-deficiency do not seem to be related in PCD induced by CD3/CD8-stimulation.

In conclusion, target KCs that express PD-L1 only at the time of inflammation protect themselves from destruction by antigen-specific CD8 T cells. Thus, upregulation of PD-L1 in skin and/or PD-1 on CD8 T cells provide novel approaches to the treatment of autoimmune mucocutaneous diseases.

4. Materials and Methods

4.1. Mice

K14-mOVA mice have been generated [17]. Rag-1-deficient OT-I mice (Taconic) were crossed with human ubiquitin C promoter-GFP transgenic mice (The Jackson Laboratory) to generate Rag-1⁻GFP⁺OT-I mice. PD-1-KO mice [9] were provided by T. Honjo (Kyoto University, Kyoto, Japan) via W. Kastenmuller (NIAID, Bethesda, MD). PD-1-KO mice and Fas-KO mice (The Jackson Laboratory) were crossed with GFP⁺OT-I mice to generate PD-1-KO and Fas-KO Rag-1⁻GFP⁺OT-I mice. All strains were on a C57BL/6 background. All animal studies were conducted with prior approval by the Animal Care and Use Committee of the NCI.

4.2. Cells

GFP⁺OT-I cells were collected from LNs of GFP⁺OT-I mice. For epidermal cells, ear and/or whole body skin were incubated for 30 min at 37°C in 0.5% trypsin (United States Biochemical) and separated into epidermis and dermis. Single epidermal cells were dissociated from the epidermal sheets in 0.05% DNase (Sigma) by pipetting and filtering. LC-depleted epidermal cells, $\gamma\delta$ T cell-depleted epidermal cells, and KCs were negatively selected from the epidermal cells using CD11c⁻, CD3⁻ and CD45⁻ MicroBeads (Miltenyi Biotec), respectively.

4.3. Adoptive transfer

Mice were injected intravenously with OT-I cells in 200 μ l RPMI1640 medium with 5% fetal bovine serum (FBS). The mice were weighed and clinically observed daily after the adoptive transfer.

4.4. Flow cytometry

SDLN cells and epidermal cells from mice and cultured OT-I cells were stained with PE-conjugated anti-CD44 (clone: IM7) and PD-L1 (MIH5), PE-CF594-conjugated anti-PD-1 (J43), CD4 (RM4-5) and CD45 (30-F11), Per-CP5.5-conjugated anti-CD62L (MEL-14), PE-Cy7-conjugated anti-Fas (Jo2) and CD3e (145-2C11), BV450-conjugated anti-TCR $\gamma\delta$ (GL3), V500-conjugated anti-I-A/I-E (M5/114.15.2), allophycocyanin (APC)-conjugated anti-V β 5 (MR9-4) and PD-L2 (TY25), mouse CD25 (PC61), AlexaFlour 700-conjugated anti-V α 2 (B20.1) and CD11c (N418), APC-Cy7-conjugated anti-CD25 (PC61) monoclonal antibodies (BD Pharmingen). LIVE/DEAD[®] fixable dead cell staining kit (violet and Near IR, Life technologies) were used to detect dead cells. Isotype matched antibodies were used as controls. Stained cells were analyzed on a LSR-II flow cytometer.

4.5. OT-I cell proliferation assay with epidermal cells

OT-I cells (1×10^5) were co-cultured with epidermal cells (1×10^5) from whole skin of naïve B6 mice or K14-mOVA mice for 2 days in RPMI 1640 with 10% FBS in flat bottom wells of 96-well plates at 37°C in a CO₂ incubator. The cells were suspended in 400 µl of PBS. GFP⁺ cells per 50 µl of the cell suspension were counted by a flow cytometer, then GFP⁺ cell numbers per well were calculated.

4.6. Knockdown of PD-L1 in KCs

KCs (1×10^5) from whole skin of naïve B6 mice or K14-mOVA mice were cultured for an hour in RPMI 1640 with 10% fetal bovine serum in 96-well flat bottom wells at 37°C in a CO₂ incubator, then added to a mixture of 160 µM PD-L1-siRNA (4 types of sequences) or control-siRNA (QIAGEN) with GenMute[®] siRNA Transfection Reagent for Primary Keratinocytes (SignaGen Laboratories). Two hours later, 20 ng/ml IFN-γ (PeproTech) or OT-I cells were added into the plates.

4.7. Measurement of cytokines

OT-I cells (4×10^5) were co-cultured with KCs (4×10^5) from whole skin of either naïve B6 mice or K14-mOVA mice for 3 days in RPMI 1640 with 10% fetal bovine serum in 24-well plates at 37°C in a CO₂ incubator. Concentrations of IFNγ and granzyme B in the culture supernatants were measured by enzyme-linked immunosorbent assay using the DuoSet[®] Kit (R&D Systems) according to the manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

K14-mOVA mouse	keratin 14 promoter-membrane ovalbumin-transgenic mouse
GVHD	graft-versus-host disease
DTg mouse	K14-mOVA/OT-I double transgenic mouse
DN T cell	double negative T cell (CD3 ⁺ B220 ⁻ CD4 ⁻ CD8 ⁻ cell)
SDLN	skin-draining lymph node
DC	dendritic cell
LC	Langerhans cell
KC	keratinocyte

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Highlights

- In a CD8 T cell-mediated mucocutaneous GVHD model, target keratinocytes upregulate PD-L1.
- PD-1-KO autoaggressive CD8 T cells cause severe autoimmune mucocutaneous GVHD-like disease.
- PD-1-KO autoaggressive CD8 T cells are activated to a great extent when they interact with target keratinocytes.
- Target keratinocytes protect themselves from destruction by antigen-specific CD8 T cells via the expression of PD-L1.

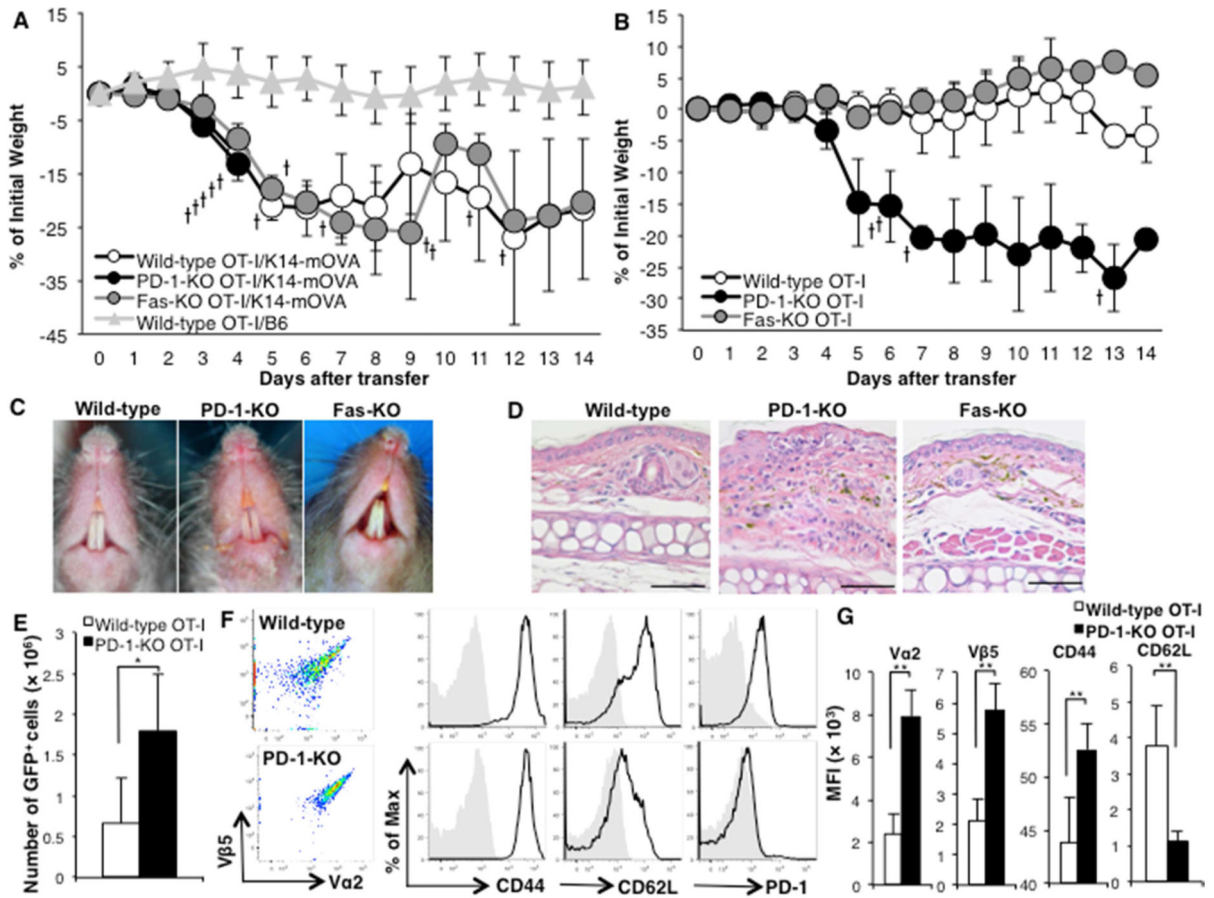


Figure 1. Adoptive transfer of PD-1-KO OT-I cells, but not wild-type or Fas-KO OT-I cells, induces severe GVHD-like disease in K14-mOVA mice

(A) GVHD-like weight loss curves of K14-mOVA mice with transfer of 1×10^6 of wild-type (open circles), Fas-KO (grey circles) or PD-1-KO (black circles) OT-I cells. Triangles represent the weight curve of B6 mice after transfer of 1×10^6 wild-type OT-I cells. (B) Weight loss curves of K14-mOVA mice following transfer of reduced numbers (5×10^4) of wild-type (open circles), Fas-KO (grey circles) or PD-1-KO (black circles) OT-I cells. The cross marks represent death of recipient mice. The bars represent SD. (C) Clinical and (D) histologic photographs of K14-mOVA mice 2 weeks after the transfer of 5×10^4 of wild-type, PD-1-KO or Fas-KO OT-I cells. (E) Cell numbers \pm SD and (F) expression of activation markers and PD-1 of transferred GFP⁺OT-I cells in skin-draining lymph nodes (SDLNs) 7 days after transfer of 5×10^4 wild-type (upper) or PD-1-KO (lower) OT-I cells. (G) The graphs show median fluorescence intensities (MFIs) \pm SD of the markers. 3 mice per group. * $P < 0.05$, ** $P < 0.01$, t test. Data is representative of 3 separate experiments.

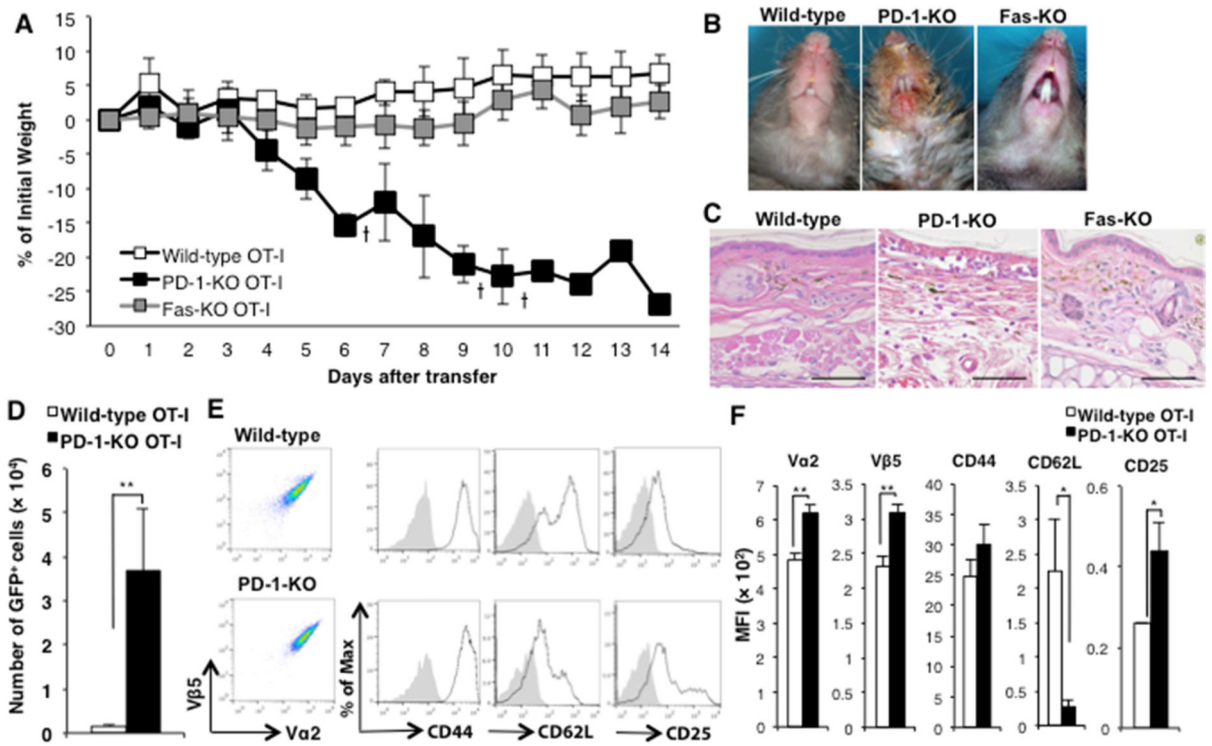


Figure 2. Transfer of PD-1KO-OT-I cells causes GVHD-like disease in K14-mOVA/OT-I double transgenic (DTg) mice, while the DTg mice are completely resistant to transfer of wild-type or Fas-KO OT-I cells

(A) GVHD-like weight loss curves of DTg mice with transfer of 1×10^6 of wild-type (open squares), Fas-KO (grey squares) or PD-1-KO (black squares) OT-I cells. The cross marks represent death of recipient mice. The bars represent SD. (B) Clinical and (C) histology photographs of DTg mice 2 weeks after the transfer of 1×10^6 of wild-type, PD-1-KO or Fas-KO OT-I cells. (D) Cell numbers \pm SD and (E) expression of activation markers of transferred GFP⁺OT-I cells in SDLNs 7 days after transfer of 1×10^6 wild-type (upper) or PD-1-KO (lower) OT-I cells. (F) MFIs \pm SD of the activation markers. 3 mice per group. ** $P < 0.01$, t test. Data is representative of 3 separate experiments.

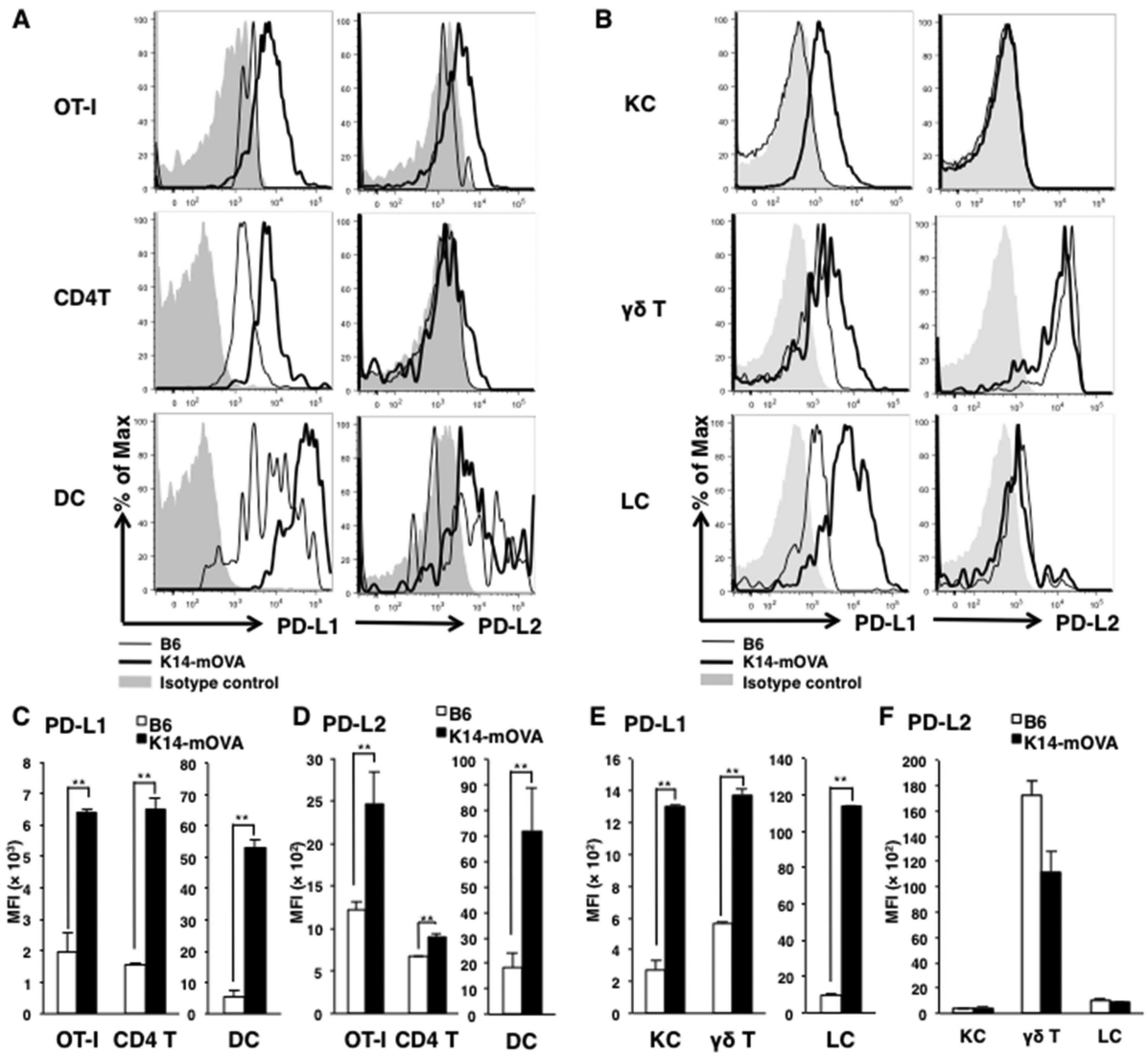


Figure 3. Immune cells and keratinocytes of mice with GVHD-like disease express PD-Ls

(A) Expression of PD-L1 and PD-L2 on indicated cell populations in SDLN cells from K14-mOVA mice (bold lines) and B6 mice (thin lines) 5 days after OT-I cell transfer were analyzed in each population. The cells were gated into OT-I cells (GFP⁺), CD4 T cells (GFP⁻CD3⁺CD4⁺), and dendritic cells (DCs, GFP⁻CD3⁻CD11c⁺I-A/I-E^{high}). (B) Expression of PD-Ls on ear skin epidermal cells from mice 10 days after OT-I cell transfer were analyzed in each population, KCs (CD45⁻), $\gamma\delta$ T cells (CD45⁺TCR $\gamma\delta$ ⁺) or Langerhans cells (LCs, CD45⁺CD11c⁺I-A/I-E^{high}). (C - F) MFIs \pm SD of PD-L1 (C - SDLN, E - ear) or -L2 (D - SDLN, F - ear) on each cell type. Open bars are cells from B6 mice and closed bars are from K14-mOVA mice. * P <0.05, ** P <0.01, t test. Data is representative of 3 separate experiments.

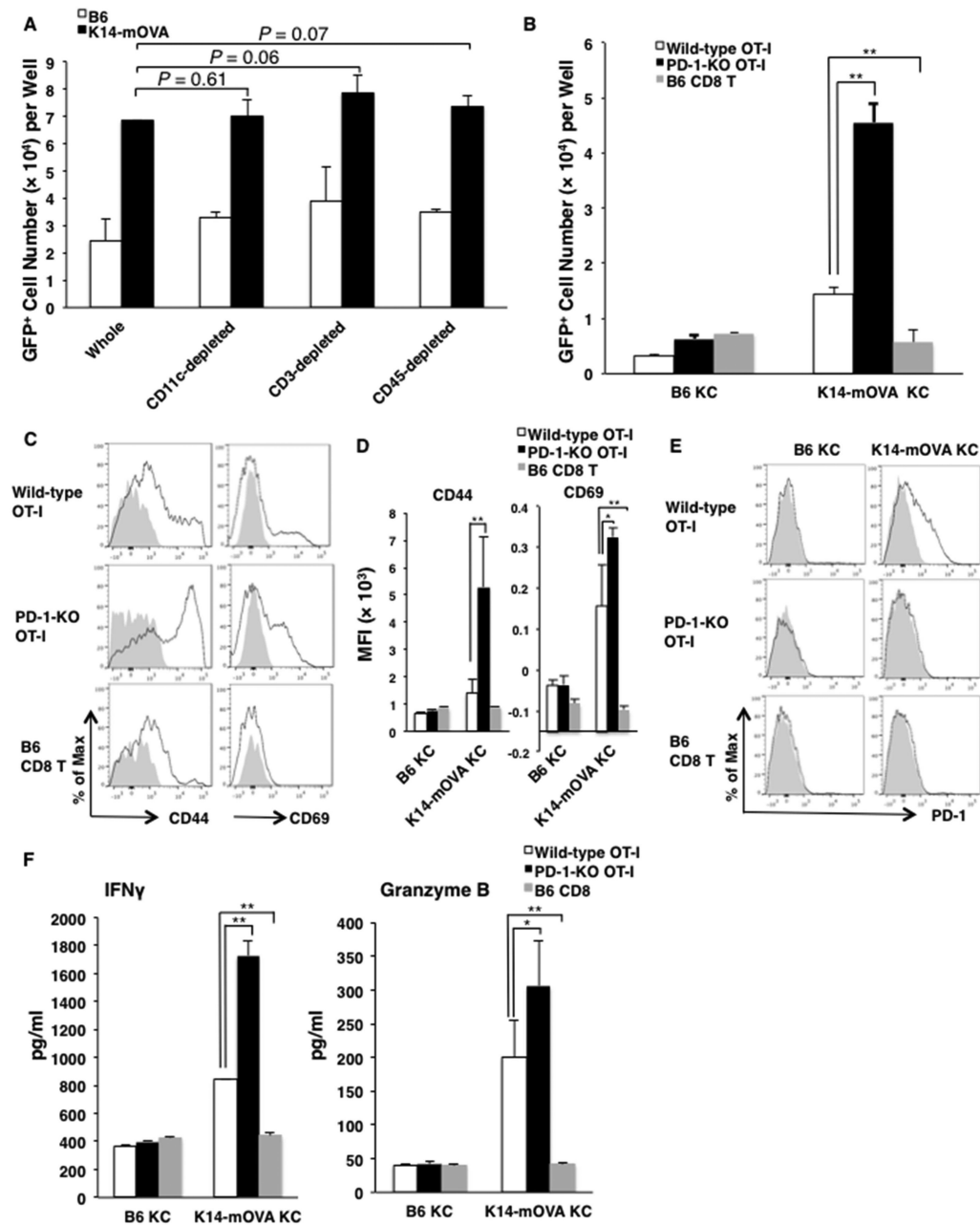


Figure 4. PD-1KO OT-I cells exhibit enhanced proliferation and activation compared to wild-type OT-I cells when co-cultured with OVA-expressing K14-mOVA keratinocytes (KC)
 (A) Whole epidermal cells (ECs), CD11c⁺cell-depleted ECs, CD3⁺cell-depleted ECs and CD45⁺cell-depleted ECs (keratinocytes; KCs) from K14-mOVA mice or B6 mice were co-cultured with GFP⁺ wild-type OT-I cells. (B) CD45⁺cell-depleted ECs (keratinocytes; KCs) from K14-mOVA mice or B6 mice were co-cultured with GFP⁺ wild-type or PD-1-KO OT-I cells, or GFP⁺ CD8 T cells. Cell numbers of the GFP⁺ cells per well were counted by flow cytometry 2 days later. The bars present means \pm SD of 3 wells. (B) Expression of CD44 and CD69 on the cultured GFP⁺ cells 3 days later. (C) MFIs \pm SD of the markers. (D) Expression of PD-1 on the GFP⁺ cells 3 days later. (E) Concentrations \pm SD of interferon

(IFN)- γ and granzyme B in culture supernatants from wells of the GFP⁺ cells co-cultured with KCs for 3 days. * P <0.05, ** P <0.01, t test. Data is representative of 4 separate experiments.

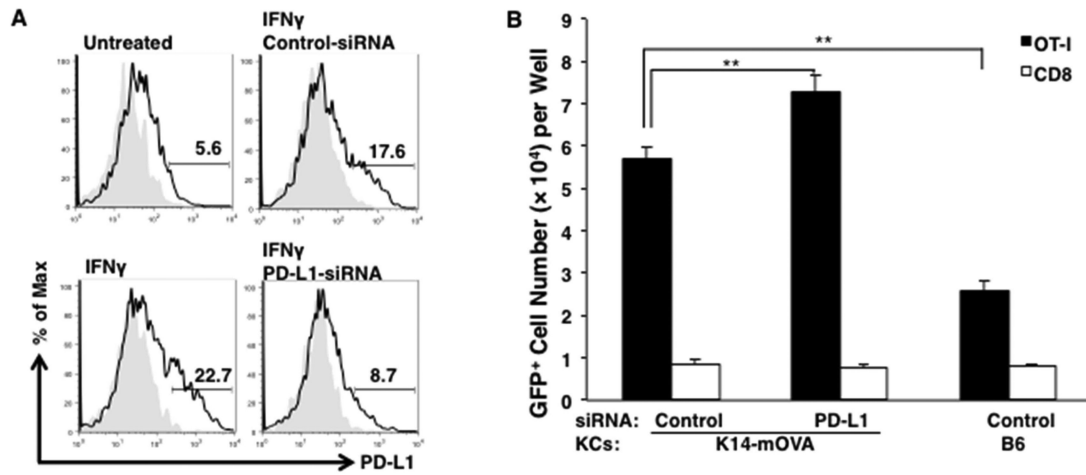


Figure 5. Knockdown of PD-L1 on K14-mOVA KCs enhances the proliferation of OT-I cells
(A) Expression of PD-L1 that KCs upregulates after stimulation of interferon (IFN)- γ was reduced by 50 percent (from 17.6 % PD-L1-positive cells to 8.7 % positive) with the transfection of PD-L1-siRNA. **(B)** GFP⁺ OT-I cells or CD8 T cells were co-cultured with KCs from K14-mOVA mice or B6 mice that were transfected with control- or PD-L1-siRNA. Numbers of the GFP⁺ cells per well were counted by flow cytometry 2 days later. The bars represent means \pm SD of 3 wells. * P <0.05, ** P <0.01, t test. Data is representative of 2 sequences and 3 separate experiments.

Table 1

Transfer of 1 million of PD-1-KO OT-I cells markedly increases serum levels of pro-inflammatory cytokines in K14-mOVA mice. Concentrations of cytokines in sera collected from K14-mOVA or B6 mice 4 days after adoptive transfer of 1×10^6 wild-type, PD-1-KO or Fas-KO OT-I cells were measured by ELISA. Averages of 4 mice \pm SD.

Recipient	OT-I cell	IFN γ	TFN α	IL-6	IL-1b
K14-mOVA	Wild-type	574.0 \pm 229.4	60.1 \pm 4.8	75.8 \pm 12.9	42.3 \pm 15.1
K14-mOVA	PD-1-KO	1527.9 \pm 599.6*	102.7 \pm 28.7*	204.2 \pm 86.4*	29.3 \pm 6.4
K14-mOVA	Fas-KO	176.5 \pm 17.4**	52.0 \pm 2.1*	52.1 \pm 2.5**	46.5 \pm 19.0
B6	Wild-type	21.2 \pm 0.3**	54.3 \pm 1.1*	31.0 \pm 4.2**	62.3 \pm 12.6

* $P < 0.05$

** $P < 0.01$ compared to the concentrations in sera from K14-mOVA mice transferred with wild-type OT-I cells, *t*-test.