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Innate IFN-γ is essential for Programmed death ligand-1-mediated T cell stimulation following *Listeria monocytogenes* infection

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Abstract

Although best characterized for sustaining T cell exhaustion during persistent viral infection, Programmed death ligand (PDL)-1 also stimulates the expansion of protective T cells after infection with intracellular bacterial pathogens. Therefore, establishing the molecular signals that control whether PDL-1 stimulates immune suppression or activation is important as immune modulation therapies based on manipulating PDL-1 are being developed. Herein, the requirement for PDL-1 blockade initiated before infection with the intracellular bacterium *Listeria monocytogenes* (Lm) in reducing pathogen-specific T cell expansion is demonstrated. In turn, the role of proinflammatory cytokines triggered early after Lm infection in controlling PDL-1-mediated T cell stimulation was investigated using mice with targeted defects in specific cytokines or cytokine receptors. These experiments illustrate an essential role for IL-12 or type I IFNs in PDL-1-mediated expansion of pathogen-specific $CD8^+$ T cells. Unexpectedly, direct stimulation by neither IL-12 nor type I IFNs on pathogen-specific $CD8^+$ cells was essential for PDL-1-mediated expansion. Instead, the absence of early innate IFN-γ production in mice with combined defects in both IL-12 and type I IFN receptor negated the impacts of PDL-1 blockade. In turn, IFN-γ ablation using neutralizing antibodies or in mice with targeted defects in IFN-γ receptor each eliminated the PDL-1-mediated stimulatory impacts on pathogen-specific T cell expansion. Thus, innate IFN-γ is essential for PDL-1-mediated T cell stimulation.

INTRODUCTION

Programmed death ligand-1 (PDL-1, B7-H1) belongs to a growing list of co-stimulation molecules within the B7 family that regulate T cell activation (1–4). Best characterized after infection with Lymphocytic choriomeningitis virus (LCMV) and other viral pathogens that cause persistent infection, stimulation via PDL-1 sustains functional exhaustion for otherwise protective viral-specific $CD8^+$ T cells (5). In turn, PDL-1 blockade using monoclonal antibodies during persistent infection or with therapeutic vaccination reinvigorates the activation of LCMV-specific $CD8^+$ T cells and accelerates pathogen eradication (6). Similarly during hepatitis B or herpes simplex virus infection, PDL-1 neutralization stimulates the activation and IFN-γ production by virus-specific T cells (7, 8). These PDL-1-mediated immune suppressive properties initially described in mouse infection models extend to functional T cell exhaustion for humans infected with viruses that predominantly cause persistent infection. For example, $CD8^+$ T cells with specificity to hepatitis C or human immune-deficiency virus each up-regulate the PDL-1 binding partner, PD-1, with progressively worsening infection (9–12). Reciprocally, PDL-1 blockade directly

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ex vivo reverses the functional exhaustion, and stimulates proliferation and cytokine production by virus-specific human $CD8^+$ T cells. Furthermore, for rabies virus that primarily cause acute instead of persistent infection, targeted defects in PDL-1 also protects against lethal infection (13). Taken together, these findings indicate PDL-1 compromises host defense against viral pathogens, and PDL-1 blockade may represent a promising strategy for boosting immunity against these infections.

Interestingly and in striking contrast to immune suppression that occurs during infection with viruses, the interaction between PDL-1 and PD-1 can also stimulate T cell activation and expansion that augments host defense against non-viral pathogens. For example, PDL-1 blockade impairs resistance and impedes the priming of protective $CD8^+$ T cells after infection with the intracellular bacterium *Listeria monocytogenes* (Lm) (14, 15). In particular, expansion defects for Lm-specific T cells with PDL-1 blockade were apparent throughout primary infection and were associated with delayed re-expansion after secondary infection (15). Similarly, mice with defects in either PDL-1 or PD-1 have blunted expansion and activation of protective $CD4^+$ T cells, and are more susceptible to other intracellular pathogens such as *Salmonella enterica* or *Mycobacterium tuberculosis* (16–18). A stimulatory role for PDL-1/PD-1 is further supported by the observation that most PD-1^{hi} $CD8^+$ T cells in healthy humans have an effector memory rather than exhausted phenotype (19). These findings illustrate that depending on the type of infection, the interaction between PDL-1 and PD-1 can provide either immune activation or suppression signals that each play important roles in controlling infection susceptibility. Therefore, establishing the specific infection-induced signals that dictate whether PDL-1 stimulates immune activation or suppression is important as immune modulation therapies based on manipulating PDL-1 are being developed.

In this study, we investigate how inflammatory cytokines induced by bacterial infection control PDL-1-mediated T cell stimulation. Given the interplay between the cytokines IL-12 and type I IFNs that each control PDL-1/PD-1 expression after infection with viral pathogens (13, 20–23), together with the efficiency whereby the intracellular bacterial pathogen Lm induces the production of these cytokines after infection (24–26), we initially focused on the role of IL-12 and type I IFNs in PDL-1-mediated stimulation of pathogen-specific T cells. Using mice with targeted individual or combined defects in these specific cytokines or their respective receptors, an essential role for either IL-12 or type I IFNs in PDL-1-mediated expansion of Lm-specific T cells is revealed. Unexpectedly however, the requirement for IL-12 and type I IFNs did not require direct stimulation by these cytokines on pathogen-specific T cells, but were instead indirectly mediated by the absence of early IFN-γ production after Lm infection in mice with combined defects in both IL-12 and type I IFN receptor. Together, these results uncover an essential role for innate IFN-γ in PDL-1-mediated T cell stimulation.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) ($CD45.2^+$ $CD90.2^+$; H-$2K^b$), Ly5.2 ($CD45.1^+$ $CD90.2^+$; H-$2K^b$), and CD90.1 ($CD45.2^+$ $CD90.1^+$; H-$2K^b$) mice were purchased from Jackson Laboratory or National Cancer Institute, and used between 6–8 weeks of age. Mice with targeted defects in PD-1, IL-12p40, type I IFN receptor, IL-12 receptor, and IFN-γ receptor, and mice with combined defects in IL-12p40 and type I IFN receptor each on the B6 background have been described (26–31). OT-1 TCR transgenic mice containing $CD8^+$ cells with specificity to the b $OVA_{257–264}$ H-2K peptide were intercrossed with WT mice (CD90.1), mice with combined defects in both IL-12 receptor and type I IFN receptor (CD90.2), or mice with targeted

defects in IFN-γ receptor (CD90.2) (32, 33). All experiments were performed under University of Minnesota IACUC approved protocols.

Lm infection

Recombinant Lm-OVA stably expresses OVA protein allowing the immune response to this surrogate Lm antigen to be tracked using established cellular immunology tools (34). To bypass differences in susceptibility to virulent Lm-OVA for mice with defects in PDL-1, specific cytokines and/or cytokine receptors, an attenuated Lm-OVA strain containing targeted defects in ΔactA that prevents intracellular and intercellular spread was used (14, 15, 24, 26, 35–39). The highly attenuated nature of ΔactA Lm results in a non-productive infection that is rapidly eliminated even in mice lacking cytokines such as IL-12 and IFN-γ required for innate resistance against virulent Lm, and normalizes the pathogen burden in IL-12 and type I IFN receptor-deficient mice where even relatively high inocula are eliminated with similar kinetics compared with control mice (26, 35, 40, 41). Similarly for anti-PDL-1 antibody treated mice, ΔactA Lm is eliminated within the first 72 hours after infection with kinetics identical to mice treated with isotype control antibody (15). For infection, Lm-OVA ΔactA was grown to early log phase (OD_{600} 0.1) in brain heart infusion media at 37°C, washed, and diluted with saline to a concentration of 10^6 CFUs per 200 μl, and this inoculum was used for intravenous injection as described (15).

Antibodies and flow cytometry

Fluorophore-conjugated antibodies and other reagents for flow cytometry were purchased from BD Bioscience (San Jose, CA) or eBioscience (San Diego, CA). For PDL-1 blockade, anti-mouse PDL-1 (10F.9G2) or rat IgG2b (LTF-2) antibodies were purchased from BioXCell (West Lebanon, NH) and injected intraperitoneally one day before (500 μg/mouse) and four days after infection (250 μg/mouse), or in separate mice beginning only four days after infection (500 μg/mouse) (5, 15). For IFN-γ neutralization, anti-mouse IFN-γ (XMG1.2) or rat IgG1 (HRPN) antibodies (BioXCell) were injected intraperitoneally one day prior to infection (1.0 mg/mouse). Lm-OVA specific $CD8^+$ T cells were identified among endogenous splenocytes by staining with $H\text{-}2K^b$ dimer X (BD Biosciences) loaded with $OVA_{257–264}$ peptide as described (15, 35). For cytokine production by antigen-specific $CD8^+$ cells, splenocytes isolated directly *ex vivo* were cultured in 96-well round-bottom plates (5×10^6 cells/ml) and stimulated with either $OVA_{257–264}$ peptide (10^{-6} M) or no stimulation in media supplemented with brefeldin A (GolgiPlug, BD Biosciences) at 37°C for 5 hours as described (15, 26, 35). For innate cytokine production, splenocytes isolated directly *ex-vivo* within the first 12, 24, and 48 hours after infection were stained using anti-IFN-γ antibody without additional stimulation. For enumerating PDL-1 and PD-1 expression, splenocytes re-suspended in saline supplemented with albumin (1%) and Fc-block (anti-CD16/32), were cell surface stained using anti-CD11c (clone N418), and anti-CD8 (clone 53.6.7), anti-PDL-1 (clones MIH5 and 1-111A) and anti-PD-1 (clone J43) antibodies.

Adoptive cell transfer

For cell transfers, 1×10^5 $CD8^+$ cells from donor WT OT-1 ($CD45.2^+$ $CD90.1^+$) mice were mixed at a 1:1 ratio with $CD8^+$ cells from either IL-12 receptor-, type I IFN receptor-deficient OT-1 ($CD45.2^+$ $CD90.2^+$), or IFN-γ receptor-deficient OT-1 ($CD45.2^+$ $CD90.2^+$) TCR transgenic mice, and intravenously transferred into recipient ($CD45.1^+$) mice one day prior to infection. For tracking the expansion of endogenous OVA-specific T cells, one mouse equivalent of $CD8^+$ cells (~10^7 cells) purified by negative selection from PD-1 deficient (CD90.2) mice were adoptively transferred into WT CD90.1 recipient mice treated with anti-PDL-1 or isotype control antibodies, and infected with Lm-OVA ΔactA the following day.

Statistics

The number and percent cells were first analyzed and found to be normally distributed, and thereafter differences between groups were evaluated using the Student's *t* test with $p < 0.05$ taken as statistical significance (GraphPad, Prism software).

RESULTS

PDL-1 blockade initiated prior to infection blunts pathogen-specific CD8$^+$ T cell expansion

Given the discordance between PDL-1 blockade prior to, compared with after, *in vivo* stimulation on CD8$^+$ cell expansion recently demonstrated for purified protein administered with poly(I:C) (42), we investigated how the timing of PDL-1 blockade would impact the expansion of pathogen-specific T cells after recombinant Lm infection. Consistent with the results of other studies (14, 15), PDL-1 blockade initiated prior to Lm-OVA infection (day −1) triggered sharp reductions in the overall expansion of OVA-specific CD8$^+$ T cells. Seven days after infection which corresponds to the peak expansion of endogenous OVA-specific CD8$^+$ T cells, ~70% reductions in OVA-specific CD8$^+$ T cells were identified both by staining with $OVA_{257–264}$ loaded H-2K^b MHC dimer or intracellular cytokine staining after stimulation with $OVA_{257–264}$ peptide for anti-PDL-1 compared with isotype antibody treated mice (Fig. 1A–D). Furthermore, this reduction in Lm-specific CD8$^+$ T cell expansion with PDL-1 blockade required PD-1, because the impacts of PDL-1 blockade were eliminated for mice with targeted defects in PD-1 in all cells or among PD-1-deficient CD8$^+$ cells after adoptive transfer into PD-1-sufficient recipients (Supplementary Figure 1). Interestingly however, when anti-PDL-1 antibody was administered later after infection (day +4), the impacts of PDL-1 blockade were reversed as OVA-specific CD8$^+$ T cells expanded ~2.5 fold more in anti-PDL-1 compared with isotype control antibody treated mice (Fig. 1A–D). Together, these results suggest the stimulatory effects of PDL-1 are controlled by signals triggered within the first few days after Lm infection.

IL-12 or type I IFNs are required for PDL-1-mediated T cell stimulation

Given the efficiency whereby Lm induces IL-12 and type I IFN production (24–26), and the importance of these cytokines in regulating PDL-1/PD-1 expression during viral infections (13, 20–23), the requirement for IL-12 or type I IFNs in PDL-1-mediated T cell stimulation were investigated with Lm infection. We found PDL-1 blockade in mice with individual defects in either IL-12 or type I IFN receptor caused significant reductions the expansion of Lm-OVA specific CD8$^+$ T cells identified by staining with either $OVA_{257–264}$ loaded H-2K^b MHC dimer or based on IFN-γ production after *in vitro* stimulation with cognate peptide similar to that observed in B6 control mice (Fig. 2A–2D). On the other hand and in sharp contrast to these reductions in Lm-OVA specific CD8$^+$ T cell expansion in B6 or mice with individual defects in IL-12 or type I IFN receptor, the impacts of PDL-1 blockade were eliminated in mice with combined defects in both IL-12 and type I IFN receptor (Fig. 2A–2D). Specifically, $OVA_{257–264}$-specific CD8$^+$ T cells identified using either $OVA_{257–264}$ H-2K^b dimer or intracellular cytokine staining each expanded to a similar extent in anti-PDL-1 or isotype antibody treated mice lacking both IL-12 production and type I IFN responsiveness. These findings illustrate the T cell stimulatory effects of PDL-1 during Lm infection require either IL-12 or type I IFNs, while the combined absence of both extinguishes PDL-1-mediated pathogen-specific T cell expansion.

Given the potential for IL-12 and type I IFNs in controlling PDL-1/PD-1 expression (13, 20–23), we also investigated the possibility that the muted impacts of PDL-1 blockade in mice with combined defects in both IL-12 and type I IFN receptor were due to differences in infection-induced PDL-1 expression. We focused initially on shifts in PDL-1 expression by CD11c$^+$ dendritic cells because these antigen-presenting cells are essential for the priming

and expansion of pathogen-specific $CD8^+$ T cells after Lm infection (43). We found although the background level of PDL-1 expression and the kinetics whereby Lm stimulates the up-regulation of PDL-1 expression by $CD11c^+$ cells each did not differ significantly in mice with individual or combined defects in IL-12 and/or type I IFN receptor compared with B6 control mice, the proportion of dendritic cells which up-regulated PDL-1 expression was reduced by ~30% in mice lacking both IL-12 production and type I IFN responsiveness (Fig. 3). Comparatively, the expression of both PD-1 and PDL-1 were enriched for Lm OVA-specific T cells compared with bulk non-specific $CD8^+$ cells, and the relative expression of each on OVA $dimer^+$ $CD8^+$ cells in mice with individual or combined defects in IL-12 and type I IFN receptor did not differ significantly from B6 control mice (Supplementary Figure 2). Nevertheless, *in vivo* treatment with anti-PDL-1 antibody clone 10F.9G2 efficiently blocked PDL-1 availability because the level of staining with other anti-PDL-1 clones (MIH5 and 1-111A) was sharply reduced for OVA-specific T cells in anti-PDL-1 compared with isotype antibody treated mice (Supplementary Figure 3). Therefore although the blunted up-regulation of PDL-1 expression by dendritic cells in mice with combined defects in IL-12 and type I IFN receptor may contribute to the negated impacts of PDL-1 blockade, the sharp up-regulation of PDL-1/PD-1 expression from background levels induced by Lm infection in mice with combined defects in both IL-12 and type I IFN receptor may also indicate important non-redundant roles for these cytokines in stimulating PDL-1-mediated pathogen-specific $CD8^+$ T cell expansion.

Cell-intrinsic stimulation by neither IL-12 nor type I IFNs are essential for PDL-1-mediated T cell expansion

Related experiments explored how IL-12 and type I IFNs may control the stimulatory effects of PDL-1. Given the potency whereby IL-12 and type I IFNs can directly stimulate T cell activation (44–47), we first investigated the requirement for cell-intrinsic stimulation with IL-12 and type I IFNs on antigen-specific T cells in overriding PDL-1-mediated T cell expansion. Specifically, we compared the impacts of PDL-1 blockade initiated one day before Lm-OVA ΔactA infection on the subsequent expansion of antigen-specific $CD8^+$ T cells from IL-12 receptor, type I IFN receptor double deficient (that cannot respond to direct cell-intrinsic stimulation by these cytokines, $CD45.2^+$ $CD90.2^+$) and WT ($CD45.2^+$ $CD90.1^+$) OT-1 TCR transgenic mice each after adoptive transfer into $CD45.1^+$ recipient mice. The discordant expression of CD90.1/90.2 congenic markers allows these two subsets of adoptively transferred antigen-specific $CD8^+$ T cells to be discriminated from each other, and expression of the CD45.2 marker allows both subsets of donor cells to be identified among recipient cells (Fig. 4A). Using this approach if cell-intrinsic stimulation via either IL-12 or type I IFNs on OVA-specific $CD8^+$ T cells is essential for PDL-1-mediated expansion, the effects of PDL-1 blockade would be eliminated for cells with combined defects in receptors for both IL-12 and type I IFNs, but preserved for WT cells that can respond to stimulation with each of these cytokines. Interestingly and in sharp contrast to this prediction, PDL-1 blockade caused reductions in expansion for both subsets of adoptively transferred antigen-specific $CD8^+$ cells each to a similar extent (~ 50%) (Fig. 4B,C). Furthermore, the magnitude of these reductions among both subsets of OVA-specific T cells each paralleled the expansion defects among endogenous OVA-specific $CD8^+$ cells with PDL-1 blockade in B6 and mice with individual defects in IL-12 and type I IFN receptor (Figs. 1 and 2) (14, 15). Together, these results demonstrate the negated impacts of PDL-1 blockade in mice with combined defects both IL-12 and type I IFN receptor are not due to defects in cell-intrinsic stimulation by IL-12 or type I IFNs on $CD8^+$ T cells. Instead, other environmental differences resulting from the combined absence of both IL-12 and type I IFNs override the immune stimulatory effects of PDL-1 following Lm infection.

Lm-induced IFN-γ production drives PDL-1-mediated T cell expansion

Since direct T cell stimulation by IL-12 and type I IFNs are jointly non-essential for PDL-1-mediated expansion of Lm-specific T cells, we investigated the role other cytokines such as IFN-γ known to be stimulated by IL-12 and type I IFNs have in controlling the T cell stimulatory impacts of PDL-1 (48–50). Consistent with the results of our prior studies (35), innate IFN-γ production by T and NK cells among bulk splenocytes that peaks within the first 12 hours after Lm-OVA ΔactA infection in B6 mice was completely abolished in mice with combined defects in both IL-12 and type I IFN receptor (Fig. 5). On the other hand, although early IFN-γ production in IL-12 deficient mice was extinguished more rapidly from peak levels compared with B6 mice, Lm-OVA ΔactA induced indistinguishable levels of IFN-γ production within the first 12 hours after infection in mice with individual defects in IL-12 or type I IFN receptor each compared with B6 mice (Fig. 5). Moreover, PDL-1 blockade did not significantly impact the magnitude or kinetics of Lm-OVA ΔactA infection-induced IFN-γ production in mice with individual or combined defects in IL-12 or type I IFN receptor each compared with B6 mice (data not shown). Thus, overriding PDL-1-mediated T cell stimulation in mice with combined defects in both IL-12 and type I IFN receptor may reflect differences in production of immune modulatory cytokines such as IFN-γ.

To investigate the requirement for IFN-γ in PDL-1-mediated T cell stimulation, the impacts of IFN-γ neutralization combined with PDL-1 blockade on pathogen-specific $CD8^+$ T cell expansion were enumerated. Remarkably, anti-IFN-γ neutralization efficiently eliminated the defects in Lm-OVA $CD8^+$ T cell expansion associated with PDL-1 blockade. Specifically, the reductions in OVA-specific T cell expansion tracked using $OVA_{257–264}$ $H\text{-}2K^b$ dimer or intracellular cytokine staining associated with PDL-1 blockade were eliminated in mice treated with anti-IFN-γ neutralizing but preserved in mice treated with each respective isotype antibody (Fig. 6A–6D). In a similar fashion, the impacts of PDL-1 blockade on pathogen-specific $CD8^+$ T cell expansion were also eliminated in mice with targeted defects in the IFN-γ receptor, but preserved in IFN-γ receptor sufficient control mice (Fig. 7A–7D). Therefore, although specific cytokine-antibody complexes have the potential to stabilize the biological activity of some cytokines *in vivo* (51–53), the paralleled elimination of PDL-1 stimulation using anti-IFN-γ neutralizing antibody and in IFN-γ receptor-deficient mice demonstrates an essential role for IFN-γ in PDL-1-mediated pathogen-specific $CD8^+$ T cell expansion after Lm infection. Together with the absence of IFN-γ production in mice with combined defects in IL-12 and type I IFN receptor (Fig. 5) (35), these findings suggest IFN-γ produced through either IL-12 or type I IFN-dependent pathways early after Lm infection dictates PDL-1-mediated expansion of pathogen-specific $CD8^+$ T cells.

Given the potential contribution of blunted PDL-1 up-regulation in negating the impacts of PDL-1 blockade in mice with combined defects in IL-12 and type I IFN receptor (Fig. 3), Lm infection-induced shifts in PDL-1 expression by $CD11c^+$ dendritic cells in IFN-γ receptor-deficient mice were also investigated. We found Lm-OVA ΔactA triggered sharply increased levels of PDL-1 expression by $CD11c^+$ dendritic cells that peaked 24 hours after infection in IFN-γ receptor deficient mice, and the overall magnitude and tempo of these shifts in PDL-1 expression were identical compared with B6 control mice (Fig. 8). Similarly, expanded Lm-OVA-specific $CD8^+$ T cells in both IFN-γ receptor deficient and B6 control mice were enriched for PD-1 and PDL-1 expression compared with bulk non-specific $CD8^+$ cells (Supplementary Figure 4). Thus, the muted impacts of PDL-1 blockade on antigen-specific $CD8^+$ T cell expansion in IFN-γ receptor deficient mice are not explained by differences in infection induced PDL-1/PD-1 expression.

Cell-intrinsic stimulation by IFN-γ is non-essential for PDL-1-mediated T cell expansion

The requirement for IFN-γ in PDL-1-mediated expansion of antigen-specific $CD8^+$ T cells following Lm infection led us to further investigate the importance of IFN-γ responsiveness by $CD8^+$ T cells, and how PDL-1 blockade may control the expansion of these cells. Using a similar adoptive transfer strategy for investigating the requirement for cell intrinsic IL-12 and type I IFN stimulation on OVA-specific $CD8^+$ cell expansion (Fig. 4), the impact of PDL-1 blockade initiated one day before Lm-OVA ΔactA infection on the subsequent expansion of antigen-specific $CD8^+$ T cells from IFN-γ-receptor-deficient ($CD45.2^+$ $CD90.2^+$) compared with WT ($CD45.2^+$ $CD90.1^+$) OT-1 TCR transgenic mice each after adoptive transfer into CD45.1 recipient mice was evaluated. We found PDL-1 blockade in recipient mice prior to Lm-OVA ΔactA infection caused reductions in the expansion for both subsets of adoptively transferred OT-1 $CD8^+$ T cells each to a similar extent (Fig. 9A–9C). Furthermore, the overall magnitude of these reductions (~ 50%) were similar to the reductions among endogenous OVA specific $CD8^+$ T cells and among adoptively transferred $CD8^+$ cells from TCR transgenic mice with defects in the receptors for both IL-12 and type I IFNs (Figs. 2 and 4). Taken together, these results indicate that although IFN-γ produced early after Lm infection is essential for PDL-1-mediated $CD8^+$ T cell expansion, and direct cell intrinsic IFN-γ stimulation on effector T cells has been described to be important for their expansion in other infections (54–57), IFN-γ stimulation on $CD8^+$ T cells is non-essential for their expansion following Lm infection.

DISCUSSION

The balance between immune stimulation and suppression signals that together control T cell activation and expansion is intricately regulated. Although PDL-1 has been mostly characterized to mediate suppression and functional T cell exhaustion during persistent viral infections (5, 9–12), the interaction between PDL-1 and PD-1 can also stimulate T cell activation most notably after infection with intracellular bacterial pathogens. For example, PD-1-deficient mice have increased pathogen burden and readily succumb to even relatively low inocula of aerosol MTb (17). Moreover during MTb infection, PD-1 expression marks highly proliferative pathogen-specific T cell precursors that replenish protective cytokine producing effector $CD4^+$ cells (17, 58). Similarly, PDL-1 disruption using mice with targeted defects in this molecule or blocking antibodies administered prior to infection, augments infection susceptibility and impedes the expansion of pathogen-specific T cells after infection with other intracellular bacterial pathogens such as *Salmonella enterica* and *Listeria monocytogenes* (14, 15, 18). Accordingly, PDL-1 stimulation has the potential to provide either immune activation or suppression signals, and these discordant roles are most likely controlled by differences in the cytokine milieu or other infection-induced environmental differences between intracellular bacterial compared with viral pathogens. Our finding that PDL-1-mediated T cell stimulation that occurs with PDL-1 blockade initiated before Lm infection is reversed with blockade initiated four days after infection (Fig. 1) further illustrates discordant roles for PDL-1 at early and later time points within the same infection. These results are consistent with the discordant impacts resulting from PDL-1 blockade initiated before compared with after *in vivo* stimulation with purified protein plus poly(I:C) on T cell expansion (42), and together reinforce the potential importance of the cytokine milieu that shifts dramatically within the first few days after acute infection or immunization on the ensuing immune response through PDL-1.

By initiating PDL-1 blockade prior to infection in mice with targeted defects in cytokines such as IL-12 and type I IFNs that are readily induced by Lm and other intracellular bacterial pathogens, we sought to identify how PDL-1-mediated T cell stimulation is controlled. Although IL-12 and type I IFNs have each been described to control PDL-1/PD-1 expression in various other contexts (13, 20–23), we found mice with individual

defects in each cytokine (or cytokine receptor) showed no significant differences in PDL-1 expression by $CD11c^+$ antigen presenting cells or PD-1/PDL-1 expression by antigen-specific $CD8^+$ T cells (Fig. 2 and Supplementary Figure 2). Interestingly however, Lm infection-induced up-regulation of PDL-1 expression was blunted in mice with combined defects in both IL-12 and type I IFNs. Although the reduced levels of PDL-1 expression in mice lacking both IL-12 production and type I IFN receptor may contribute to the negated impacts of PDL-1 blockade, the requirement for IFN-γ in PDL-1-mediated T cell expansion of Lm-OVA specific $CD8^+$ T cells suggests these results are more likely explained by the absence of IFN-γ triggered early after Lm infection in these mice. In turn, the comparable magnitude and tempo whereby PDL-1 expression is up-regulated in IFN-γ receptor-deficient and control mice indicates the requirement for IFN-γ in PDL-1-mediated T cell expansion are not simply due to differences in PDL-1 expression. Instead, other IFN-γinduced molecules such as nitric oxide that are up-regulated with PDL-1 blockade and suppress T cell proliferation may explain the requirement for IFN-γ in PDL-1-mediated T cell stimulation (59, 60). This notion is supported by the sharp up-regulation of reactive nitrogen intermediates after Lm infection that occurs in an IFN-γ–dependent fashion (60, 61), and suggest establishing how PDL-1 controls nitric oxide production after in vivo infection are important areas for future investigation.

The requirement for IFN-γ in PDL-1-mediated T cell expansion during infection with intracellular bacterial pathogens is consistent with the essential role this cytokine plays in host defense against these infections (40, 62, 63). Our results suggest that during infection with these pathogens, early IFN-γ, in addition to activating innate host defense, also promotes the expansion of protective pathogen-specific T cells via PDL-1. This association between intracellular pathogens that stimulate a Tc1/Th1-dominated response and the reversal of PDL-1 from immune suppression to activation signals are also consistent with T-bet-mediated repression of PD-1 expression that stimulates the activation of viral specific $CD8^+$ T cells (64). Reciprocally, for other pathogens like LCMV clone 13 or *M. bovis* BCG that cause persistent infection and where early IFN-γ is less critical for innate host defense, PDL-1 stimulates T cell suppression instead of activation signals (5, 65–67). A notable exception here is *Toxoplasma gondii* infection where IFN-γ is critically required for innate protection, and yet PDL-1 actively inhibits protective $CD8^+$ T cell responses (68, 69). Whether this is unique for *Toxoplasma* or more generalizable for other parasitic pathogens remains undefined, but also represent important areas for future investigation. Finally, given the discordant roles in host defense resulting from PDL-1/PD-1 disruption, developing PDL-1 blockade for therapeutically boosting immunity against some infections need to be carefully weighed against the potential for increased susceptibility to infection with other types of pathogens. The finding that early IFN-γ is essential for PDL-1-mediated T cell stimulation suggests PDL-1 disruption will most severely impede host defense against infections where IFN-γ is produced early and presumably essential for innate resistance. Our ongoing studies are aimed at further investigating the interplay between PDL-1 and IFN-γ in stimulating pathogen-specific adaptive responses using other models of experimental infection, and dissecting the molecular basis whereby IFN-γ redirects PDL-1 stimulation into T cell activation signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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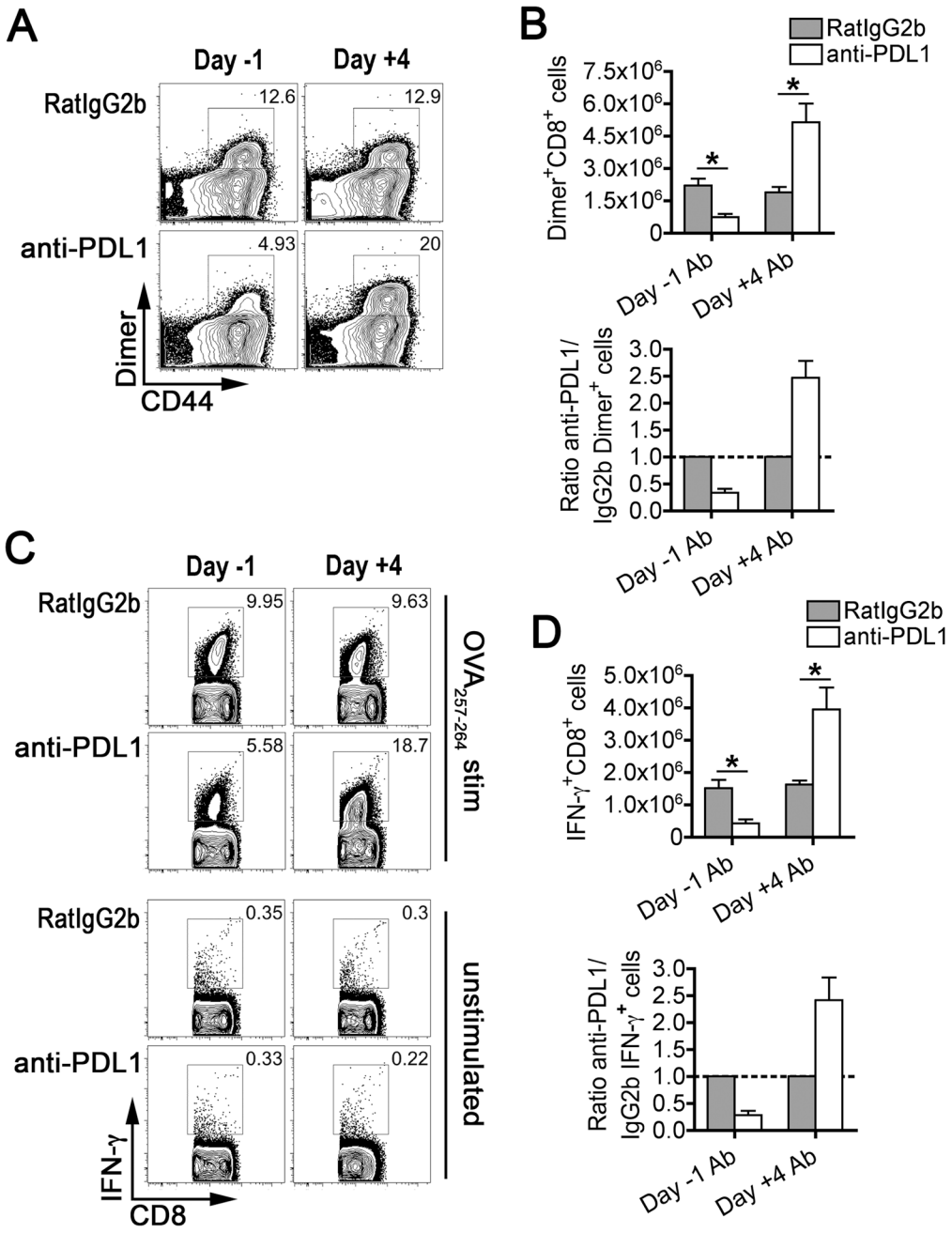

Figure 1.

PDL-1 blockade initiated prior to infection blunts pathogen-specific $CD8^+$ T cell expansion. A. Percent OVA-specific $CD8^+$ splenocytes identified by H-$2K^b$ $OVA_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for B6 mice treated with anti-PDL-1 or isotype control (IgG2b) antibody initiated either one day before (day −1) or four days after (day +4) infection. B. Total number (top) and ratio (bottom) of OVA $dimer^+$ $CD8^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing $CD8^+$ splenocytes seven days after Lm-OVA ΔactA infection, and $OVA_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for B6 mice treated with anti-PDL-1 or isotype control antibody initiated either one day before (day −1) or four days after (day +4) infection. D. Total number (top)

and ratio (bottom) of IFN-γ producing $CD8^+$ T cells for the mice described in panel C. These data reflect 7–12 mice per group from three independent experiments each with similar results. Bar, one standard deviation. * $p < 0.05$.

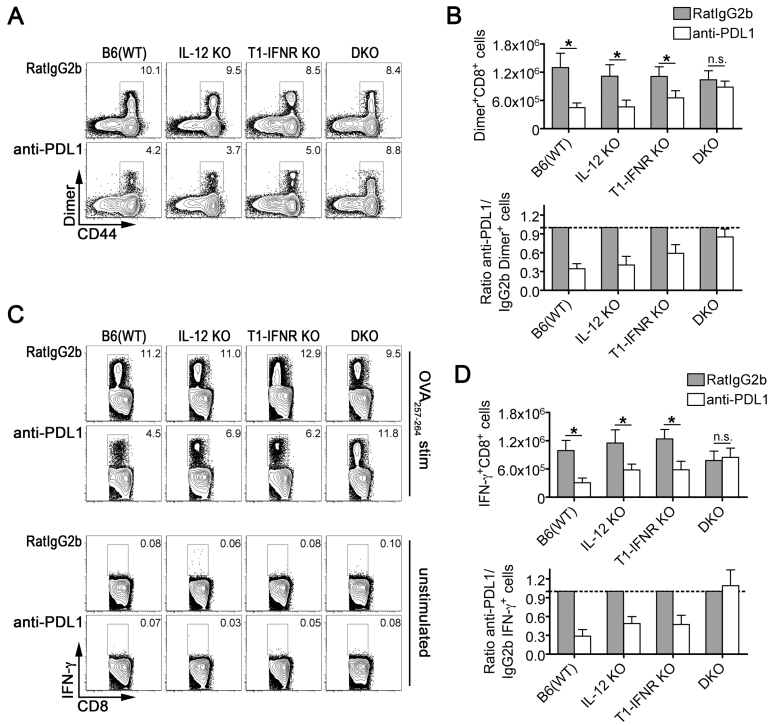

Figure 2.

IL-12 or type I IFN receptor is required for PDL-1-mediated expansion of pathogen-specific $CD8^+$ T cells. A. Percent OVA-specific $CD8^+$ splenocytes identified by H-$2K^b$ $OVA_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for each group of mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA-specific $dimer^+$ $CD8^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing $CD8^+$ splenocytes seven days after Lm-OVA ΔactA infection, and $OVA_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for each group of mice treated with either anti-PDL-1 or isotype antibody one day prior to infection. D. Total number (top) and ratio (bottom) of IFN-γ producing $CD8^+$ T cells for the mice described in panel C. These data reflect 7–12 mice per group from three independent experiments each with similar results. Bar, one standard deviation, * $p < 0.05$.

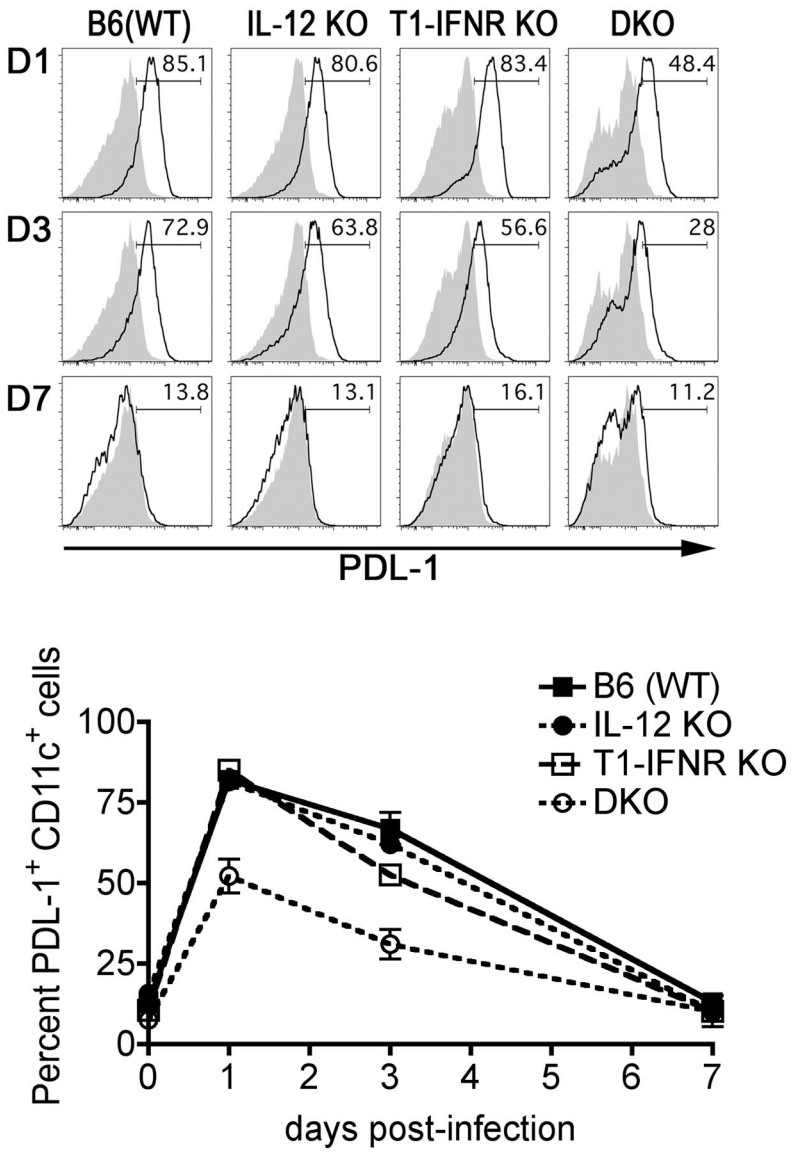

Figure 3.

Infection-induced shifts in PDL-1 expression by $CD11c^+$ antigen presenting cells. Representative plots (top) and composite data (bottom) illustrating PDL-1 expression by $CD11c^+$ splenocytes for each group of mice at the indicated time points after Lm-OVA ΔactA infection (line histogram) compared with uninfected controls (shaded histogram). These data reflect 6–8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

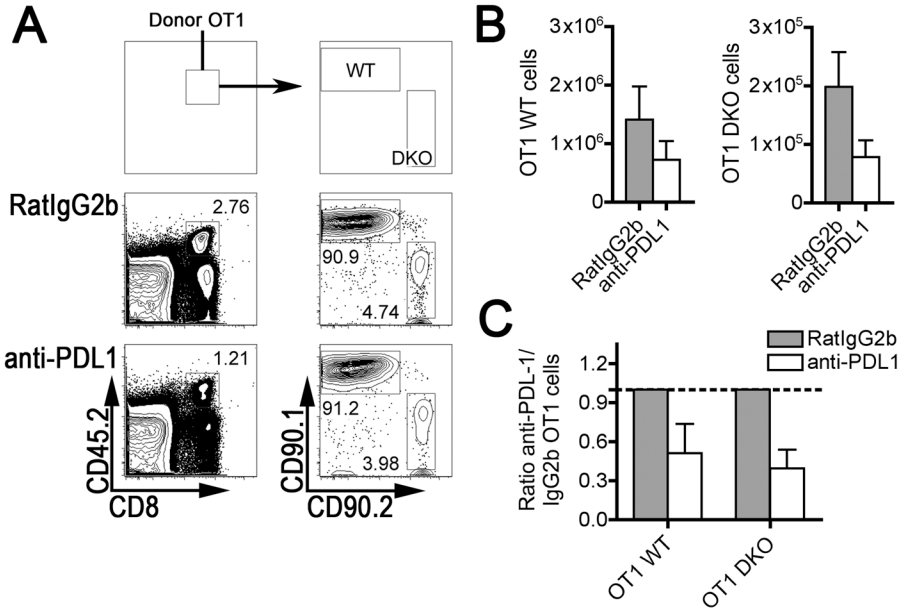

Figure 4.

T cell-intrinsic stimulation by neither IL-12 nor type I IFNs are essential for PDL-1-mediated pathogen-specific $CD8^+$ T cell expansion. A. Percent donor ($CD45.2^+$) $CD8^+$ cells among either WT ($CD90.1^+$) or IL-12 receptor, type I IFN-receptor DKO ($CD90.2^+$) OT-1 cells three days after Lm-OVA ΔactA infection, for cells adoptively transferred into CD45.1 recipient mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day before infection. Total number (B) and ratio (C) of adoptively transferred OVA-specific $CD8^+$ T cells from WT or IL-12 receptor, type I IFN-receptor DKO OT-1 transgenic mice for the mice described in panel A. These data reflect 6–8 mice per group from three independent experiments. Bar, one standard deviation.

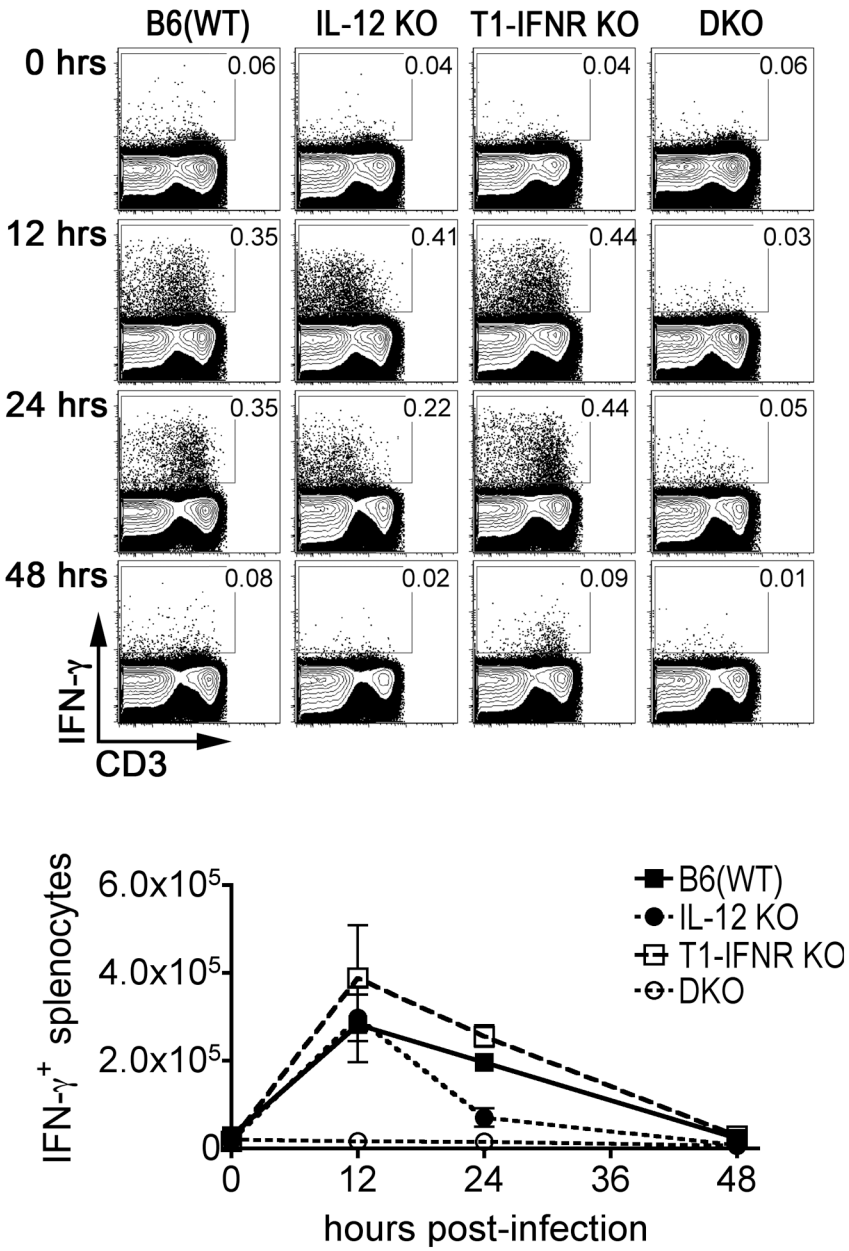

Figure 5.

IL-12 or type I IFNs are essential for innate IFN-γ production after Lm infection. Representative FACS plots illustrating percent (top) and composite data demonstrating total number (bottom) of IFN-γ producing splenocytes for each group of mice at the indicated time points after Lm-OVA ΔactA infection. These data reflect 6–8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

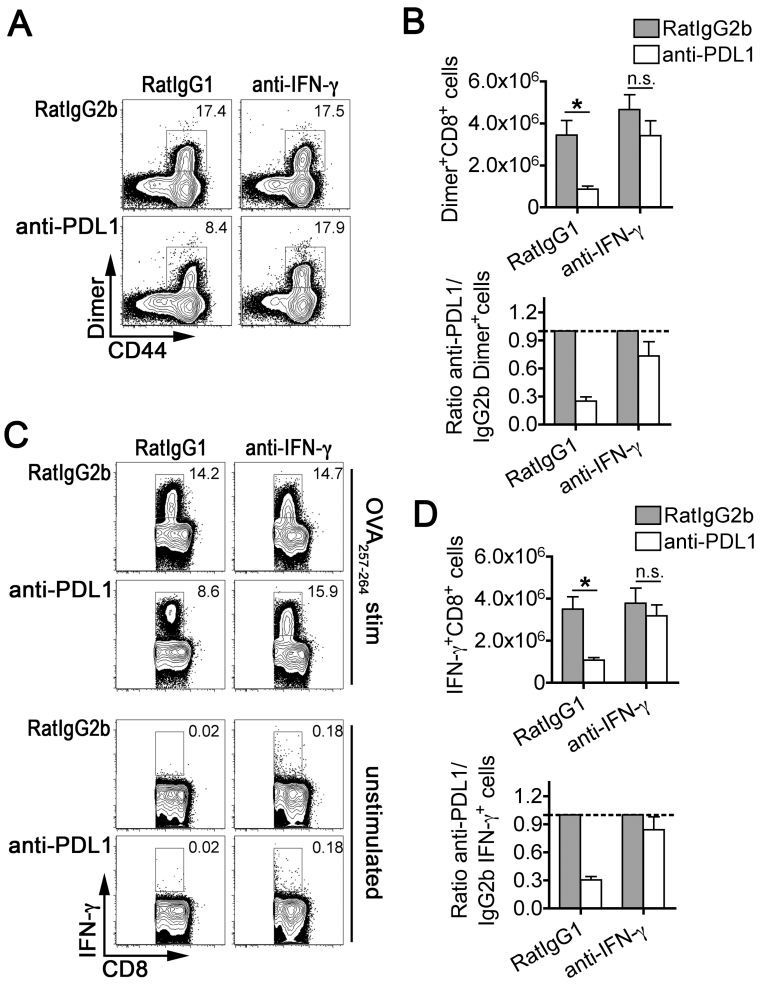

Figure 6.

IFN-γ neutralization eliminates PDL-1-mediated expansion defects for pathogen-specific $CD8^+$ T cells. A. Percent OVA-specific $CD8^+$ splenocytes identified by H-$2K^b$ $OVA_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for B6 mice treated with anti-PDL-1, and/or anti-IFN-γ, or each respective isotype control antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA $dimer^+$ $CD8^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing $CD8^+$ splenocytes seven days after Lm-OVA ΔactA infection, and $OVA_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for each group of mice, treated with anti-PDL-1, and/or anti-IFN-γ, or each respective isotype control antibody one day prior to infection. D. Total number (top) and ratio (bottom) of IFN-γ producing $CD8^+$ T cells for the mice described in panel C. These data reflect 7–12 mice per group from three independent experiments each with similar results. Bar, one standard deviation. * $p < 0.05$.

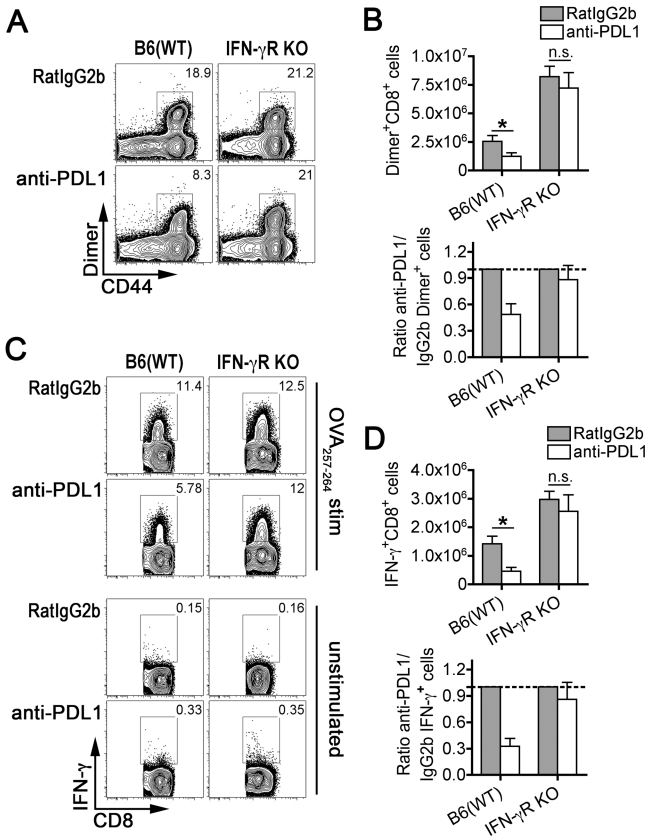

Figure 7.

Expansion defects for pathogen-specific $CD8^+$ T cells with PDL-1 blockade are eliminated in IFN-γ receptor-deficient mice. A. Percent OVA-specific $CD8^+$ splenocytes identified by H-$2K^b$ $OVA_{257–264}$ dimer staining seven days after Lm-OVA ΔactA infection for B6 (WT) or IFN-γ-receptor-deficient mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day prior to infection. B. Total number (top) and ratio (bottom) of OVA $dimer^+$ $CD8^+$ T cells for the mice described in panel A. C. Percent IFN-γ producing $CD8^+$ splenocytes seven days after Lm-OVA ΔactA infection, and $OVA_{257–264}$ peptide stimulation (top) or no stimulation controls (bottom) for each group of mice treated with anti-PDL-1 or isotype (IgG2b) antibody one day prior to infection. D. Total number (top) and ratio (bottom) of IFN-γ producing $CD8^+$ T cells for the mice described in panel C. These data reflect 7–10 mice per group from three independent experiments each with similar results. Bar, one standard deviation. * $p < 0.05$.

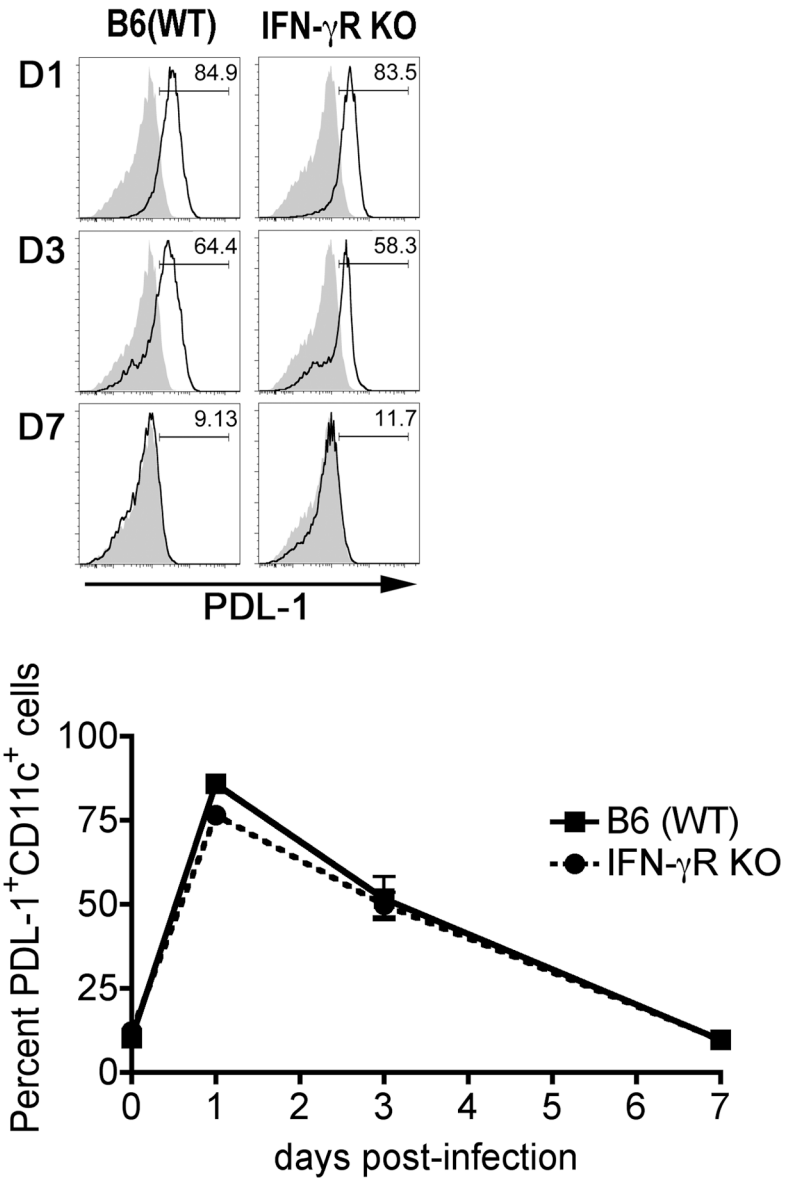

Figure 8.

IFN-γ receptor plays non-essential roles for infection-induced up-regulation of PDL-1 expression. Representative plots (top) and composite data (bottom) illustrating PDL-1 expression by $CD11c^+$ splenocytes for each group mice at the indicated time points after Lm-OVA ΔactA infection (line histogram) compared with uninfected controls (shaded histogram). These data reflect 6–8 mice per group from three independent experiments each with similar results. Bar, one standard deviation.

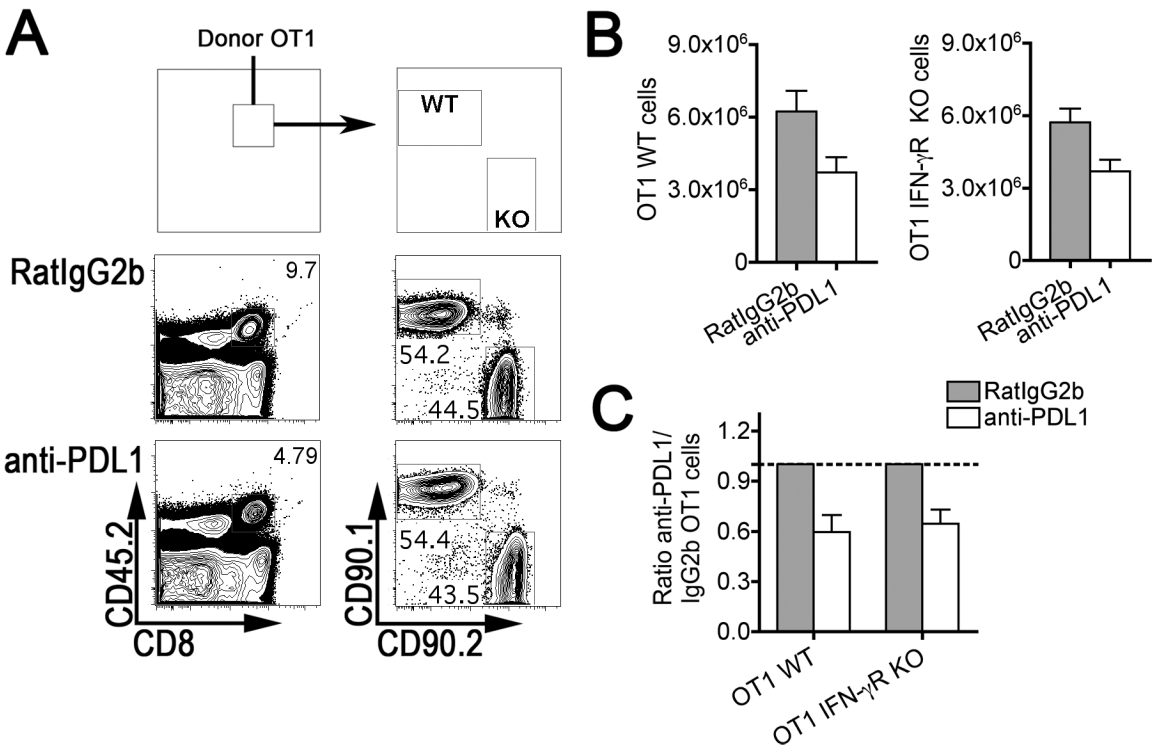

Figure 9.

T cell-intrinsic stimulation by IFN-γ is non-essential for PDL-1-mediated expansion of pathogen-specific $CD8^+$ T cells. A. Percent donor ($CD45.2^+$) $CD8^+$ T cells among WT ($CD90.1^+$) or IFN-γ receptor-deficient ($CD90.2^+$) OT-1 cells three days after Lm-OVA ΔactA infection, for cells adoptively transferred into CD45.1 recipient mice treated with either anti-PDL-1 or isotype control (IgG2b) antibody one day before infection. Total number (B) and ratio (C) of adoptively transferred OVA-specific $CD8^+$ T cells from WT or IFN-γ receptor-deficient OT-1 transgenic mice for the mice described in panel A. These data reflect 6 mice per group from three independent experiments each with similar results. Bar, one standard deviation.