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Blockade of Programmed Death-1 (PD-1) in young (New Zealand Black × New Zealand White)F_1 mice promotes the suppressive capacity of CD4+ regulatory T cells protecting from lupus-like disease

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Abstract

PD-1 usually acts as a negative signal for T cell activation, and its expression on $CD8^+Foxp3^+$ T cells is required for their suppressive capacity. Here we show that PD-1 signalling is required for the maintenance of functional regulatory $CD4^+CD25^+Foxp3^+$T cells ($CD4^+T_{reg}$) that can control autoimmunity in (New Zealand Black × New Zealand White) F_1 (BWF_1) lupus mice. PD-1 signalling induced resistance to apoptosis and prolonged the survival of $CD4^+T_{reg}$. *In vivo*, the blockade of PD-1 with a neutralizing antibody (Ab) reduced PD-1 expression on $CD4^+T_{reg}$ ($PD1^{lo}CD4^+T_{reg}$). $PD1^{lo}CD4^+T_{reg}$ had an increased ability to promote B cell apoptosis and to suppress $CD4^+$ helper T cells (T_h) as compared to $CD4^+T_{reg}$ with elevated PD-1 expression ($PD1^{hi}CD4^+T_{reg}$). When PD-1 expression on $CD4^+T_{reg}$ was blocked *in vitro*, $PD1^{lo}CD4^+T_{reg}$ suppressed B cell production of IgG and anti-dsDNA Ab. Finally, *in vitro* studies showed that the suppressive capacity of $CD4^+T_{reg}$ depended on PD-1 expression, and that a fine tuning of the expression of this molecule directly affected cell survival and immune suppression. These results indicate that PD-1 expression has multiple effects on different immune cells that directly contribute to a modulation of autoimmune responses.

Introduction

In SLE, uncontrolled lymphocyte autoreactivity is a result of a breach of tolerance to self. In the BWF_1 lupus murine model, protection from clinical disease is influenced significantly by both $CD4^+CD25^+Foxp3^+$ regulatory T cells ($CD4^+T_{reg}$) and inhibitory $CD8^+CD28^-Foxp3^+$ T cells ($CD8^+T_i$) that suppress autoantibody (autoAb) production (1, 2).

Here we explore the influence of the programmed cell death-1-programmed death-1 ligand (PD-1/PD-L1) pathway in $CD4^+T_{reg}$ of BWF_1 mice that are predisposed to the development of lupus-like disease.

PD-l/PD-L1 is one of the costimulatory pathways that regulate the balance between stimulatory and inhibitory signals for self-tolerance (3). In particular, PD-1 plays an important role in maintaining T-cell tolerance by maintaining the unresponsiveness of effector T cells (T_{eff}) (4). Different mechanisms involving PD-1/PD-L1 signaling are in place to induce and maintain tolerance at different sites, at different times, and within different T-cell populations, including $CD4^+T_{reg}$. PD-1 signaling in $CD4^+T_{reg}$ may play a

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role in affecting their function so that $CD4^+T_{reg}$ can restrain the numbers of Ag-reactive T_{eff} that accumulate in response to an immunogenic stimulus (5). PD-1 signaling counteracts the downstream activation biochemical cascade after activation via TCRs in T_{eff}. This signaling also slows cell trafficking of circulating $CD4^+T_{reg.}$ However, inhibition of PD-1 in $CD4^+T_{reg}$ may have different outcomes, depending on the Ag-stimulation in their target T_{eff}.

We previously showed that the induction of immune tolerance following administration of the Ig-related peptide pConsensus (pCons) in BWF_1 mice induced two suppressive T cell populations, $CD4^+CD25^+Foxp3^+$ and $CD8^+Foxp3^+$ T_{reg} The $CD8^+T_{reg}$ had reduced expression of PD-1 as compared to untreated controls (2). In addition, blockade of PD-1 *in vivo* protected young BWF_1 mice from developing lupus-like disease, due in part to an increase in the suppressive activities of $CD8^+$ T cells (6), suggesting that PD-1 favored the emergence of inhibitory $CD8^+$ T cells. Since $CD8^+$ T cells are targets of $CD4^+T_{reg}$-mediated suppression but also influence the activity of $CD4^+T_{reg}$, it is relevant to understand the role of PD-1 expression in the regulatory activity of $CD4^+T_{reg}$, i.e., in their ability to suppress $CD4^+CD25^-$ helper T cells (T_h) and B cells.

Here we report that, in contrast to naïve BWF_1 mice in which the percentage of $CD4^+T_{reg}$ declines over time, anti-PD-1 treatment *in vivo* preserves functional suppressive $Foxp3^+CD4^+CD25^+$ T cells for several weeks. PD-1 expression is inversely correlated with Foxp3 expression in $CD4^+T_{reg}$, and the expression of low levels of PD-1 on $CD4^+T_{reg}$ promotes their regulatory capacity. $PD1^{lo}CD4^+T$ (compared to $PD1^{hi}CD4^+T_{reg}$) had increased TGF-β production and were resistant to apoptosis. A moderate reduction of PD-1 expression in $CD4^+T_{reg}$ allowed the $CD4^+T_{reg}$ to induce B cell apoptosis and to suppress T_h proliferation, while very low levels of PD-1 expression resulted in a loss of the regulatory capacity of $CD4^+T_{reg}$. These data suggest that PD-1 expression modulates the suppressive function of $CD4^+T_{reg}$ in a quantitative manner, and that an effective function of $CD4^+T_{reg}$ depends on low, but not absent, expression of PD-1.

Materials and Methods

Mice

NZB ($H\text{-}2^{d/d}$), NZW ($H\text{-}2^{z/z}$) and NZB/W F_1 ($H\text{-}2^{d/z}$) (BWF_1) mice and C57BL/6 (B6) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were treated in accordance with the guidelines of the UCLA Animal Research Committee, an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experiments were conducted in female mice.

Antibody (Ab) treatment

10 week-old mice were treated with intraperitoneal injections of 100 μg of anti-PD-1 Ab (Clone J34, Armenian hamster, eBioscience, San Diego, CA), or 100 μg of control isotype-matched IgG (Clone 2Bio299Arm, Armenian hamster, eBioscience), every other day for total of three injections. The anti-PD-1 Ab inhibits the binding of PD-1 by PD-L1 on cells as tested by the manufacturer, but it does not induce either apoptosis or stimulation in cells that express PD-1.

Cell isolation and staining

One week after mAb administration, blood was obtained from the retroorbital vein. After lysis of red blood cells with ACK lysing buffer (Sigma, St. Louis, MO), PBMC were centrifuged, washed and resuspended in PBS for flow cytometry analysis. For splenocytes, single cell suspensions of splenocytes were prepared by passing cells through a sterile wire mesh before resuspension in HL-1 medium (BioWhittaker, Walkersville, MD). In

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experiments involving whole cell populations, $CD4^+$ and B cells were isolated by positive selection via magnetic bead separation (anti-L3T4 and anti-CD19 respectively, Miltenyi Biotec, Auburn, CA), and $CD4^+CD25^+$, $CD4^+CD25^-$ cells were separated by selection with beads prior to anti-$L3T4^+$ cell isolation using biotnylated-anti-CD25 followed by anti-biotin beads on an AutoMACS® System (Miltenyi). Populations were >95% pure by subsequent FACS analysis.

FACS analysis

Phenotypic analysis of splenocytes and PBMCs from the mice was performed with a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) using CellQuest (BD Biosciences). Staining with multiple combinations of Ab (indicated in the pertinent sections) was performed according to standard procedures described elsewhere (2). The Abs used were purchased from eBioscience and BD Biosciences. FACS analysis was performed by Flowjo (Tree Star, Inc., Ashland, OR).

Intracellular Staining

For intracellular staining, cells were first stained for expression of cell surface markers and then fixed, permeabilized, and stained using the Cyto-fix/cytoperm kit (eBioscience) according to manufacturer's instructions. Intracellular cytokines (Foxp3) were identified in cells that had been fixed and permeabilized by staining with the appropriate Abs. Intracellular staining was performed using Abs obtained from eBioscience and Biolegend.

Cell Cultures

Methods have been described previously (2, 7). In brief, purified $CD4^+CD25^-$ T_h from 16-week old, unmanipulated BWF_1 females, and $CD19^+$ B cells from older unmanipulated BWF_1 females with high titers of anti-DNA in serum, were cultured with syngeneic $CD4^+CD25^+$ T cells ($CD4^+T_{reg}$ hereafter) from control or treated mice in 96-well microtiter plates. With 5×10^4 $CD4^+T_{reg}$ in each plate, $CD4^+T_{reg}$ were cocultured with T_h and B cells at a ratio of 10:10:1, at 37° C in complete medium containing antibiotics and 10% fetal calf serum for 72 hours. We previously determined that these are optimal ratios to detect suppression of T_h and of B (2). Unless otherwise stated, mouse anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) mAb were added to cultures at the start of incubation to activate $CD4^+$ T cells. Supernatants were collected after 72 hours of incubation, and used for measurements of cytokines and anti-dsDNA antibodies by standard ELISA. Cells were analyzed for cell proliferation (see below).

Detection of apoptosis

Splenocytes were resuspended in apoptosis buffer (BD Biosciences), according to the manufacturer's instructions, and incubated with 7-aminoactinomycin D (7-AAD) and PE-conjugated Annexin V (eBioscience) for 15 min at room temperature. The percentage of $Annexin^+7AAD^-$ cells was determined by flow cytometry.

Measurement of cell proliferation

Splenocytes were isolated from BWF_1 mice at age 25 weeks for culture *in vitro* as described above. $CD4^+CD25^-$ T_h were incubated for 10 minutes at 37°C with 5uM CFSE (Invitrogen, Grand Island, NY). Cells were washed with medium containing 10% FBS, cocultured with $CD19^+$ B cells and activated $CD4^+CD25^+$ T_{reg} for 3 days, followed by staining with CD4-PerCP Ab (eBioscience). Cell proliferation of T_h was measured by flow cytometry analyzed by Flowjo, which was expressed as division index (the average number of cell divisions that the responding cells underwent) calculated by the software.

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Measurement of anti-DNA and cytokines in supernatants of cell cultures

IgG anti-DNA was measured in the supernatant of cultured spleen cells and PBMC, and in serum using BD OptEIA Enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences), with the mean absorbance read at 410/490 nm. Cytokine measurement was done using commercial ELISA kits (eBioscience and Biolegend).

Statistical analyses

Statistical analyses were performed using Prism 4 software (GraphPad, San Diego, CA). Comparisons between two groups were performed by the two-tailed t-test. Tests between more than two groups were performed by one-way analysis of variance (ANOVA). In cases where repeated measures were assessed, two-way ANOVA and mixed effects ANOVA models were used. Tukey's multiple comparison test (one-way ANOVA) and Bonferroni posttest (two-way ANOVA) were used in post-hoc testing between pairs of groups. p values < 0.05 were considered significant. Our papers on $CD4^+T_{reg}$ in the BWF_1 mouse model in the past used 6 mice in each group for *in vitro* experiments yielding statistically significant data (2, 6). With the sample size of 6 mice in each group, the minimally detectable (with 80% power) effect size (difference in means divided by the standard deviation) is 1.33 assuming a two group t-test with a 0.05 two-sided significance level. The magnitude of the minimally detectable effect was smaller than that observed in the pilot studies, suggesting that this sample size was sufficient.

Results

PD-1 blockade *in vivo* promotes the generation of adaptive regulatory $CD4^+CD25^+$ T cells

$CD4^+T_{reg}$ play an important role in maintaining immune tolerance in BWF_1 mice (8). Here we studied the influence of PD-1 expression in $CD4^+T_{reg}$ by using PD-1 blockade *in vivo* with a neutralizing Ab against PD-1, starting when BWF_1 and B6 mice were 10 weeks old.

In IgG isotype-treated control BWF_1 mice, the percentage of $CD4^+T_{reg}$ in PBMC decreased markedly over time. In contrast, mice with anti-PD-1 treatment maintained the percentage of $CD4^+T_{reg}$ between 10 and 20 weeks of age (Fig. 1A, $p < 0.01$). Fewer $CD4^+T_{reg}$ from BWF_1 mice with anti-PD-1 treatment expressed PD-1 when compared to controls (Fig. 1B, $p < 0.01$), with a significantly decreased PD-1 expression measured by MFI (Fig. 1D, $p < 0.01$). We refer thereafter to the $PD1^+CD4^+T_{reg}$ derived from (*in vivo*) anti-PD-1 treated mice as $PD1^{lo}CD4^+T_{reg}$, and those from IgG isotype controls as $PD1^{hi}CD4^+T_{reg}$ (Fig. 1E). B6 mice were similar to BWF_1 mice in that as the IgG-treated control mice aged, the percentage of $CD4^+T_{reg}$ decreased (Fig. 1A, $p < 0.001$), and *in vivo* PD-1 blockade reduced the decreased percentage of $CD4^+T_{reg}$. On the other hand, PD-1-expressing $CD4^+T_{reg}$ in control B6 mice were significantly increased when compared to anti-PD-1 Ab-treated mice (Fig. 1C, $p < 0.004$).

Among the $CD4^+T_{reg}$, there was increased Foxp3 expression within 3 weeks in BWF_1 mice after treatment with anti-PD-1 Ab (Fig. 1F, $p < 0.01$). These $CD4^+CD25^+Foxp3^+$ cells were $Helios^-$, suggesting that they are adaptive regulatory T cells. Foxp3 expression was inversely related to PD1 expression in $CD4^+T_{reg}$; as mice aged, Foxp3 expression decreased as the level of PD-1 rose (Fig. 1B, 1F). At the age of 25 weeks, when $CD4^+T_{reg}$ from anti-PD-1 treated mice had significantly less PD-1 expression compared to Ig-treated controls, a higher percentage of stimulated $PD1^{lo}CD4^+T_{reg}$ expressed Foxp3 (Fig. 1G, $p < 0.05$).

Taken together, these data suggest that in unmanipulated B6 and BWF_1 mice, PD-1 expression in $CD4^+T_{reg}$ increases with age. Also, Foxp3 expression is inversely related to the expression of PD-1 in the $CD4^+T_{reg}$.

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Altered PD-1 signaling *in vivo* increases the regulatory capacity of $CD4^+T_{reg}$ in BWF_1 mice

Decreased apoptosis in T_{eff} and B cells has been linked to clinical autoimmunity, and $CD4^+T_{reg}$ can induce apoptosis in T_h or B cells. Here we examined the role of $CD4^+T_{reg}$ in regulating the susceptibility of their target cells to apoptosis, when $CD4^+T_{reg}$ from mice treated *in vivo* with anti-PD-1 were cocultured with T_h and B cells.

$PD1^{lo}CD4^+T_{reg}$ were resistant to apoptosis as compared to $PD1^{hi}CD4^+T_{reg}$ (Fig. 2A, 2B, $p < 0.05$). In contrast, syngeneic B cells in coculture with $PD1^{lo}CD4^+T_{reg}$ had increased apoptosis (Fig. 2C, $p < 0.001$, Fig. 2D, $p < 0.05$). Similarly, co-culturing with $PD1^{lo}CD4^+T_{reg}$ followed by activation of T_h increased apoptosis and limited cell proliferation (Fig. 2E, 2F, $p < 0.001$).

The tolerogenic effect of PD-1 blockade on $CD4^+T_{reg}$ is dose-dependent

$CD4^+T_{reg}$ from spleens of 20 week old IgG isotype-treated or anti-PD-1-treated BWF_1 mice were cultured with 0, 25, 50, 75 and 100 μg/ml of anti-PD-1 *in vitro* for 72 hours. Treatment of $PD1^{lo}$ and $PD1^{hi}$ $CD4^+T_{reg}$ with anti-PD-1 *in vitro* decreased the surface expression of PD-1 (Fig. 3A).

As shown in Fig 2, $PD1^{lo}CD4^+T_{reg}$, compared to $PD1^{hi}CD4^+T_{reg}$, were resistant to apoptosis, and they were more effective in suppressing T_h proliferation and in inducing B cell apoptosis. In Fig. 3B, with the addition of anti-PD-1 Ab *in vitro*, $CD4^+T_{reg}$ from both groups of mice were more resistant to apoptosis than without anti-PD-1 Ab ($p < 0.05$), but the percentage of apoptotic $CD4^+T_{reg}$ levelled off in both groups at about 15%. $PD1^{lo}CD4^+T_{reg}$ apoptosis levelled off at a lower concentration of *in vitro* anti-PD-1 Ab (50 μg/ml) than that of $PD1^{hi}CD4^+T_{reg}$ (75 μg/ml). This observation suggests that PD-1 blockade at a moderate level helps to maintain the survival of $CD4^+T_{reg}$, but protection from apoptosis plateaus at higher concentrations of PD-1 blockade. Incidentally, the higher concentrations of anti-PD-1 did not cause cell death of $CD4^+T_{reg}$ directly, as tested by cell staining with trypan blue (not shown), and there was no evidence for $CD4^+T_{reg}$ stimulation at any concentration measured as cell division index by CFSE (not shown).

Next, we looked at the relationship between the concentration of anti-PD-1 Ab and the suppressive capacity of $CD4^+T_{reg}$. $PD1^{hi}CD4^+T_{reg}$ required anti-PD-1 Ab to improve their suppressive effect. At an anti-PD-1 Ab concentration ≤ 50 μg/ml, $PD1^{lo}CD4^+T_{reg}$ were resistant to apoptosis and suppressed T_h proliferation (Fig. 3B, 3C), induced B cell apoptosis (Fig. 3D) and reduced IgG production by B cells (Fig. 3E). However, $PD1^{lo}CD4^+T_{reg}$ began to lose their suppressive function with anti-PD-1 Ab at >50 μg/ml, which was associated with increased T_h cell division, decreased B cell apoptosis and increased IgG production. $PD1^{hi}CD4^+T_{reg}$ manifested enhanced regulatory effects *in vitro* with anti-PD-1 Ab up to 75 μg/ml, and this effect levelled off or was diminished when anti-PD-1 Ab concentration was >75 μg/ml. An explanation for the difference in the suppressive capacity between $PD1^{lo}CD4^+T_{reg}$ and $PD\text{-}1^{hi}CD4^+T_{reg}$ is that lower PD-1 expression in $CD4^+T_{reg}$ promotes suppression (see comparison of baseline numbers in Figs. 3 B-E). However, in the case of $PD1^{lo}CD4^+T_{reg}$ with PD-1 blockade *in vitro*, $CD4^+T_{reg}$ with very low or near absent PD-1 expression can lose their suppressive capacity. Thus, the degree of PD-1 blockade can be important in maintaining viability and function of $CD4^+T_{reg}$.

PD-1 blockade preserves regulatory function of $CD4^+T_{reg}$ *in vitro*

We reported that in mice treated with anti-PD-1 *in vivo*, serum levels of IFN-γ (primarily from T_h), total IgG, and IgG anti-dsDNA (from B cells) were significantly decreased (9). If suppression results in anergy, T_h may have lower proliferation or decreased production of IFN-γ, and B cells may have lower Ig and/or proinflammatory cytokine production. To

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examine the direct effect of anti-PD-1 on $CD4^+T_{reg}$, we treated 22-week old unmanipulated BWF_1 splenic $CD4^+T_{reg}$ with 75 μg/ml of anti-PD-1 Ab (to generate $PD1^{lo}CD4^+T_{reg}$), or matched Ig isotype (to generate $PD1^{hi}CD4^+T_{reg}$) for 24 hrs followed by washing, before coculturing with unmanipulated syngeneic T_h and B cells, and assessed the production of autoAb *in vitro*. Significantly less IFN-γ, IL-6 and IL-10 were produced in the cocultures containing $PD1^{lo}CD4^+T_{reg}$ as compared to $PD1^{hi}CD4^+T_{reg}$, whether $CD4^+T_{reg}$ were alone or stimulated and with T_h plus B (Fig. 4A-C, $p < 0.001$). Total IgG and anti-DNA Ab production were significantly decreased in the supernatants of cultures containing $PD1^{lo}CD4^+T_{reg}$ (Fig. 4D, 4E, $p < 0.001$).

PD-1 blockade increased IL-2 expression to induce the expansion of $CD4^+T_{reg}$

Low concentrations of IL-2 are required for maintaining the suppressive capacity of $CD4^+T_{reg}$ (10), but high levels of IL-2 reverse it (10, 11). PD-1-mediated signals generally inhibit IL-2 production, and the addition of IL-2 restores the responses (12-14). We previously reported that the decline in serum levels of IL-2, which is characteristic of BWF_1 mice with aging, occurred significantly later in mice treated with anti-PD-1 Ab (9), and IL-2 could overcome PD-1-mediated inhibition if $CD4^+T_{reg}$ can produce IL-2 *en route* to tolerance. Here we found that even though the percentage of $IL\text{-}2^+CD4^+CD25^-$ T cells declined as animals aged, numbers were sustained in anti-PD-1 treated mice at 30 weeks of age (data not shown). Moreover, $CD4^+T_{reg}$ from 22-week old unmanipulated mice were cultured *in vitro* with 75 μg/ml anti-PD-1 Ab to generate $PD1^{lo}CD4^+T_{reg}$, followed by neutralizing anti-IL-2 mAb, washed and cocultured with unmanipulated, syngeneic T_h and B cells. As reported in Figs. 3 and 4, $PD1^{lo}CD4^+T_{reg}$ suppressed T_h proliferation and IgG production in coculture but after treatment with anti-IL-2 Ab, their suppressive effect was abrogated (Supplemental Fig. S1). Next, we repeated the experiment by pre-treating $PD1^{lo}CD4^+T_{reg}$ with IL-2 prior to coculture. Interestingly, while the suppressive function of $PD1^{lo}CD4^+T_{reg}$ on T_h proliferation and IgG production was preserved with the addition of IL-2 at 5 U/ml, suppression was lost at 20 U/ml (data not shown). These data suggest that the demand for IL-2 in $CD4^+T_{reg}$ is altered upon PD-1 blockade.

The suppressive effect of $CD4^+T_{reg}$ induced by anti-PD-1 depends on TGF-ß but not IL-6

Our previous work showed that serum TGF-ß levels were increased in anti-PD-1-treated BWF_1 mice when compared to IgG isotype controls (9), and that the upregulation of Foxp3 expression in $CD4^+T_{reg}$ depended on their TGF-ß production (2). To test whether the suppressive effect of PD-1 on $CD4^+T_{reg}$ was dependent on TGF-ß, we repeated the *in vitro* coculture experiment setup described in Fig 4, with $PD1^{lo}CD4^+T_{reg}$ exposed to a blocking Ab against TGF-β *in vitro* prior to coculture with T_h and B cells.

In the presence of antagonistic anti-TGF-β Ab, $PD1^{lo}CD4^+T_{reg}$ decreased their Foxp3 expression, increased their PD-1 expression and increased apoptosis (Fig. 5A-C, $p< 0.001$). The ability of $PD1^{lo}CD4^+T_{reg}$ to suppress IL-6, IL-10 and IgG production was lost (Fig. 5D-F, $p < 0.001$).

It has been shown that IL-6 can abrogate the suppressive effect of $CD4^+T_{reg}$ in a DC-dependent manner (15-17). When combined with TGF-β, IL-6 can stimulate T_{h17} differentiation from naïve $CD4^+$ T cells, whereas IL-6 inhibits the generation of $CD4^+T_{reg}$ induced by TGF-β (18, 19). We blocked IL-6 with an inhibitory Ab *in vitro* similar to our testing of TGF-β. The suppressive effect of $PD1^{lo}CD4^+T_{reg}$ on IgG production was not significantly affected by IL-6 blockade (Fig. 5G).

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In vivo blockade of PD-1 in autoAb-producing BWF_1 mice delayed disease progression

We previously reported that *in vivo* blockade of PD-1 in young BWF_1 mice significantly delayed appearance of autoAbs and proteinuria, and prolonged survival (9). To understand whether downregulation of PD-1 after the onset of disease can facilitate $CD4^+T_{reg}$ in diseased mice, we exposed BWF_1 to neutralizing anti-PD-1 Ab at the age of 22 weeks, when mice have circulating autoAbs (such as anti-dsDNA Ab and high total IgG) and mild proteinuria <2+ by urinary dipstick (9). Age-matched unmanipulated animals were compared to mice treated with anti-PD-1 or control IgG isotype Ab for the development of proteinuria and for survival. Interestingly, mice with "late" anti-PD-1 treatment had delayed onset of proteinuria (Fig. 6A, $p< 0.0001$) and improved survival (Fig. 6B, $p < 0.0001$) compared to control mice, but the delay was less than in mice treated early before disease was manifest clinically. These data suggest that PD-1 blockade favors a delay in disease progression during early stages of active disease but less so when the disease has already advanced.

Discussion

This manuscript shows that PD-1 signalling promotes survival and function of adaptive $CD4^+T_{reg}$ in this model of murine lupus.

The levels of expression of PD-1 are important in regulating the function of $CD4^+T_{reg}$. $CD4^+T_{reg}$ suppress T_h and autoAb-producing B cells when PD-1 expression is intact and neither too high nor too low, and different levels of expression or engagement of PD-1 can cause different immune responses in $CD4^+T_{reg}$.

PD-1/PD-L1 interactions downregulate T cell activation under some circumstances, and PD-1 or PD-L1 expression on peripheral tissues and T cells can be crucial to protect from autoimmunity (20, 21). Experiments from different autoimmune disease mouse models (22-24) and immune-driven conditions (25) have shown enhanced severity and/or disease onset upon disruption of the PD-1/PD-L interaction. Despite this finding, few studies have examined the role of PD-1 during early activation of naïve T cells or during the development of T-cell tolerance (26). Our data show that effector $CD4^+$ T cells targeted by $PD1^{lo}CD4^+T_{reg}$ were impaired in their ability to proliferate and to produce cytokines, and they were more susceptible to apoptosis when compared to similar cells from naïve age-matched BWF_1 females. It is acknowledged that the changes in cytokine levels in co-culture supernatants cannot only be attributed to $CD4^+T_{reg}$, yet blockade of PD-1 promoted $CD4^+T_{reg}$ function. Adaptive $CD4^+T_{reg}$ with low levels of PD-1 in BWF_1 mice had higher Foxp3 expression and increased suppressive capacity *in vitro* (27), and blocking PD-1 expanded $CD4^+T_{reg}$. Also, the phenotype of $CD4^+CD25^+Foxp3^+Helios^-$ suggested that $PD\text{-}1^{lo}CD4^+T_{reg}$ are adaptive regulatory T cells generated in the periphery. Our findings are consistent with those by with Sharpe et al. that showed that PD-L1 synergizes with TGF-β in promoting conversion of naïve $CD4^+$ T cells into $Foxp3^+CD4^+T_{reg}$ (28), since TGF-β antagonistic Ab abrogated the generation of $PD1^+Foxp3^+CD4^+T_{reg}$ and blunted suppression of autoAb production.

In regard to IL-10, this cytokine can stimulate B-cell proliferation and enhance the production of anti-dsDNA Ab and immune complexes which, in turn, induce IL-10 production (29, 30). Since PD-1 and PD-L1 induce IL-10 production (31), one of the mechanisms of how blockade of PD-1 decreases anti-dsDNA production and immune complex formation our model could be by reducing IL-10 production in $CD4^+T_{reg}$ and B cells.

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IL-2 could play a role in tolerance by expanding suppressive $CD4^+T_{reg}$ and by protecting $CD4^+T_{reg}$ from apoptosis. The downregulation of PD-1 upregulated Foxp3 expression in $CD4^+T_{reg}$, possibly by interacting with NFAT (NFATc1 is a critical factor for PD-1 induction in T cells (32)), and repressed the induction of IL-2. With the PD-1 signal already attenuated by the blocking Ab, IL-2 stimulation might override PD-1-mediated inhibition and render effector cells refractory to $CD4^+T_{reg}$ suppression (yet the fact that manipulation of IL-2 *in vitro* may affect T_h proliferation makes it difficult to attribute those observations only to changes in $CD4^+T_{reg}$ function).

It seems unexpected that blockade of the PD-1 molecule promotes regulatory T cell activities, when PD-1 is under most conditions a co-inhibitory receptor on T cells. It is our hypothesis that variance in quantities of PD-1 and/or strength of signalling and/or degree of ligation of PD-1 cause different effects on the functions of $CD4^+T_{reg}$. However, it is possible that the mAb used in our experiments to block PD-1 has agonistic activity in the induction of $CD4^+T_{reg}$. Another possible explanation is that the costimulatory network of the PD-1 signalling pathway is complicated by the presence of additional receptors for PD-L1 and PDL2 that can deliver costimulatory signals (33, 34). The bidirectional signalling pathways make PD-L1 both a receptor and ligand, so that it can induce both stimulatory and inhibitory signals based on the circumstances. In the cancer model, PD-1 can deliver a reverse signal through PD-L1 to induce an anti-apoptotic effect (35). PD-L1 can engage B7-1 to deliver an inhibitory signal to T cells, and induce T cell suppression *in vitro* and T cell tolerance *in vivo* (36, 37). In our study, we suspect that the probable ablation of PD-1/PD-L1 interaction by blocking PD-1 receptor directs PD-L-1 to bind B7-1, and promotes positive costimulatory signalling through CD28, which allows increased inhibitory functions of $CD4^+T_{reg}$.

Overall, these data show that low levels of PD-1 expression in $CD4^+T_{reg}$ promote their suppressive functions, which are linked to Foxp3 and IL-2 expression and to TGF-β. These results can be relevant for the manipulation of regulatory T cell responses in SLE and related targeted immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

SLE	systemic lupus erythematosus
BWF_1	(New Zealand Black × New Zealand White)F_1
PD-1	programmed death-1
PD-L1	programmed death-1 ligand 1
Ab	antibody
mAb	monoclonal antibody
pCons	pConsensus

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TCR	T cell receptor
T_h	T helper cells
T_i	suppressive/inhibitory T cells
T_{reg}	regulatory T cells

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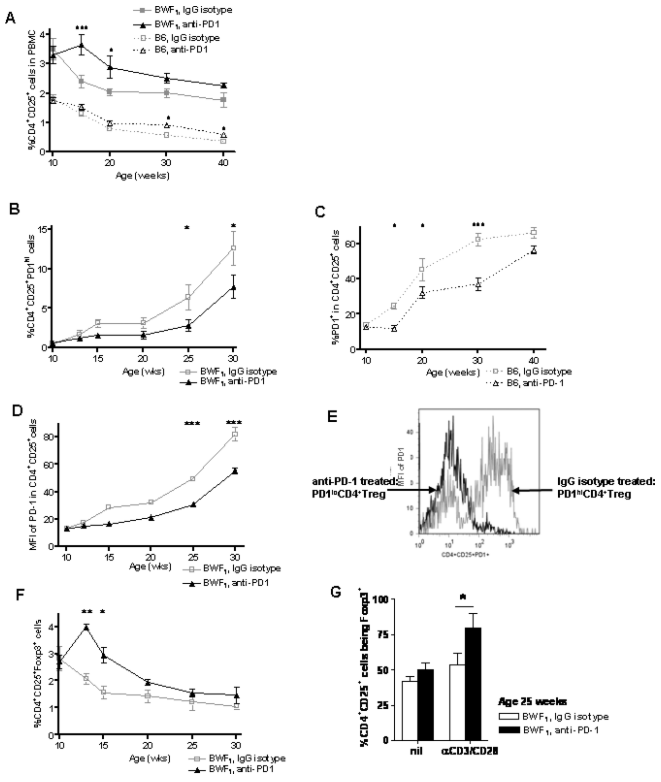

Fig 1. PD-1 blockade in vivo was associated with increased Foxp3 expression and decreased PD-1 expression in $CD4^+CD25^+$ T cells

(A) PD-1 blockade attenuated the decrease in percentage of $CD4^+CD25^+$ cells with age. At a young age (10 week old) of BWF_1 and B6 mice, *in vivo* PD-1 blockade maintained the percent of $CD4^+T_{reg}$ at a higher level than in control mice (BWF_1 and B6, $p < 0.01$, two-way ANOVA). (B) Percentage of PD-1^{hi} $CD4^+CD25^+$ cells went up with age in BWF_1 mice, but anti-PD-1 treated mice expressed fewer $PD1^{hi}CD4^+T_{reg}$ when compared to control mice. (C) Fewer cells expressed PD-1 in B6 compared to BWF_1 mice. (D) MFI for PD-1 expression in $CD4^+CD25^+$ cells was higher in BWF_1 mice treated with anti-PD-1 *in vivo* than in mice treated with IgG isotype (Fig. 1 A-D, $p < 0.01$, two-way ANOVA). (E) Representative histogram of the MFI for PD-1 in $CD4^+CD25^+PD1^+$ cells from an anti-PD1-treated BWF_1 mouse vs. an IgG isotype-treated control (at age 16 weeks) showing that PD-1 expression was reduced by treatment with anti-PD-1 Ab. We referred hereafter to the $PD1^+CD4^+T_{reg}$ derived from (*in vivo*) anti-PD-1 treated mice as $PD1^{lo}CD4^+T_{reg}$, and those from IgG isotype controls as $PD1^{hi}CD4^+T_{reg}$. (F) Expression of Foxp3 in $CD4^+CD25^+$ cells went down with age in BWF_1 mice, but anti-PD-1 treated mice had a higher Foxp3 expression than control mice until after age 15 weeks (Fig. 1F, $p < 0.01$, two-way ANOVA). (G) There was significant increase in the percent of $CD4^+CD25^+Foxp3^+T_{reg}$ from mouse spleens at age 22 weeks after in vivo PD-1 blockade upon stimulation of TCR ($p < 0.05$, unpaired student t-test). * $p < 0.05$, ** $p < 0.01$ (Bonferroni posttest). B6, n = 6 mice per group, BWF_1, n = 14 mice per group.

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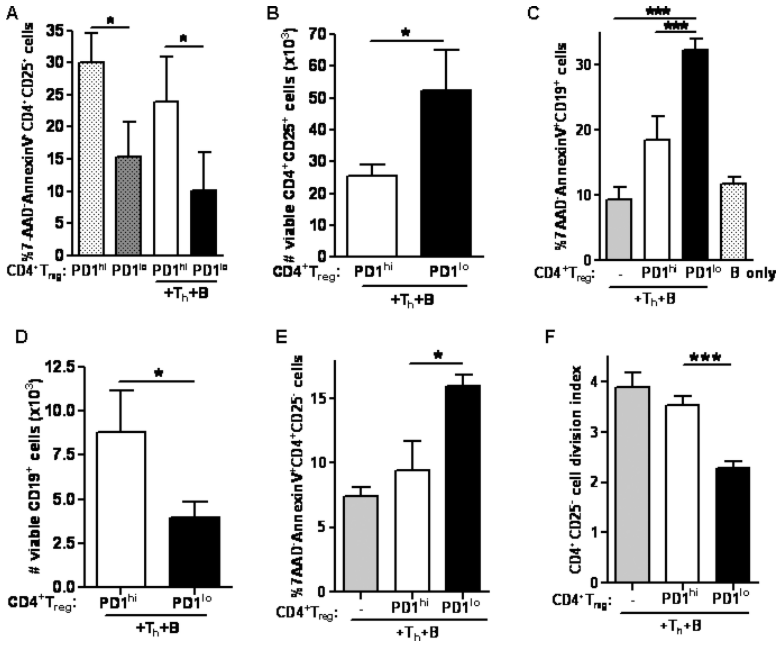

Fig 2. Inhibition of PD-1 in vivo increased the regulatory capacity of BWF_1 $CD4^+T_{reg}$ in different spleen cell subsets

(A) Apoptosis in $PD1^{lo}CD4^+T_{reg}$ was significantly less compared to $PD1^{hi}CD4^+T_{reg}$, whether or not cultures contained T_h and B cells ($p < 0.05$, unpaired student t-test). (B) $PD1^{lo}CD4^+T_{reg}$ were viable and were increased in numbers compared to $PD1^{hi}CD4^+T_{reg}$ ($p < 0.05$, unpaired student t-test). (C) $PD1^{lo}CD4^+T_{reg}$ induced more apoptosis in $CD19^+$ B cells than did $PD1^{hi}CD4^+T_{reg}$. (D) Numbers of viable B cells were decreased in cultures containing $PD1^{lo}CD4^+T_{reg}$ compared to those with $PD1^{hi}CD4^+T_{reg}$ ($p < 0.05$, unpaired student t-test). (E) $PD1^{lo}CD4^+T_{reg}$ induced more apoptosis in $CD4^+$CD25-T cells than did $PD1^{hi}CD4^+T_{reg}$. (F) $PD1^{lo}CD4^+T_{reg}$ suppressed proliferation of $CD4^+$CD25-syngeneic T cells as detected by CFSE (Fig. 2C, 2E, 2F, $p < 0.001$, one-way ANOVA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's test). Data are representative of 2 individual experiments, n = 6 mice per group in each experiment.

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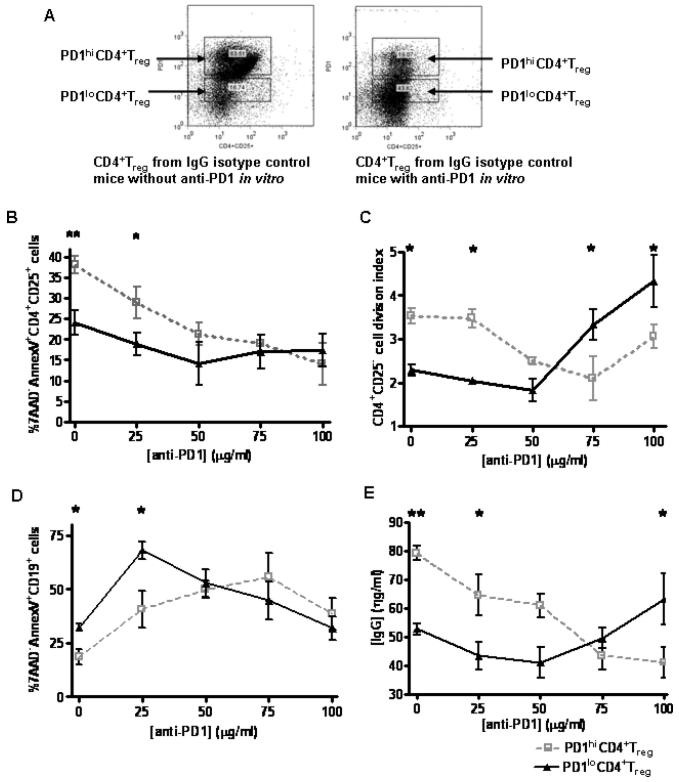

Fig 3. Effects of PD-1 blockade on the suppressive function of $CD4^+T_{reg}$ depends on the concentration of anti-PD-1 Ab to which the $CD4^+T_{reg}$ are exposed

$CD4^+T_{reg}$ were obtained from BWF_1 mice treated with anti-PD-1 Ab *in vivo* ($PD1^{lo}CD4^+T_{reg}$, black solid line), or isotype IgG ($PD1^{hi}CD4^+T_{reg}$, gray dashed line). PD-1 blockade of these $CD4^+T_{reg}$ was tested by pretreatment of harvested splenic $CD4^+T_{reg}$ *in vitro* with different concentrations of anti-PD-1 Ab, followed by coculture of washed $CD4^+T_{reg}$ with syngeneic T_h and B cells. (A) Representative dot plot of $CD4^+T_{reg}$ from an IgG isotype control mouse before and after treatment with anti-PD-1 Ab at 75 μg/ml. Note that in Fig. 3B-E, at pretreatment of $CD4^+T_{reg}$ with zero anti-PD-1 Ab, there were expected differences between $PD1^{hi}CD4^+T_{reg}$ compared to $PD1^{lo}CD4^+T_{reg}$. The addition of low concentrations of anti-PD-1 Ab *in vitro* induced (B) increased resistance of $CD4^+T_{reg}$ to apoptosis, (C) increased the ability of $CD4^+T_{reg}$ to suppress proliferation of T_h (D) increased the ability of $CD4^+T_{reg}$ to induce B cell apoptosis, and (E) increased the ability of $CD4^+T_{reg}$ to suppress IgG production. In general, these effects of *in vitro* anti-PD-1 Ab pretreatment on $PD1^{lo}CD4^+T_{reg}$ were not detectable at anti-PD-1 Ab concentrations >50 μg/ml. In contrast, the effects of *in vitro* anti-PD-1 on $PD1^{hi}CD4^+T_{reg}$ were seen at both low and high concentrations, up to anti-PD-1 Ab concentration > 75 μg/ml (Fig. 3B, $PD1^{hi}$ versus $PD1^{lo}$ $CD4^+T_{reg}$, $p < 0.02$, anti-PD-1 concentration, $p < 0.005$; Fig. 3C-E, anti-PD-1 concentration, $p < 0.05$, interaction, $p < 0.005$, two-way ANOVA). $^*p < 0.05$, $^{**}p < 0.01$ (Bonferroni posttest). Data are representative of 2 individual experiments, n = 5 mice per group in each experiment.

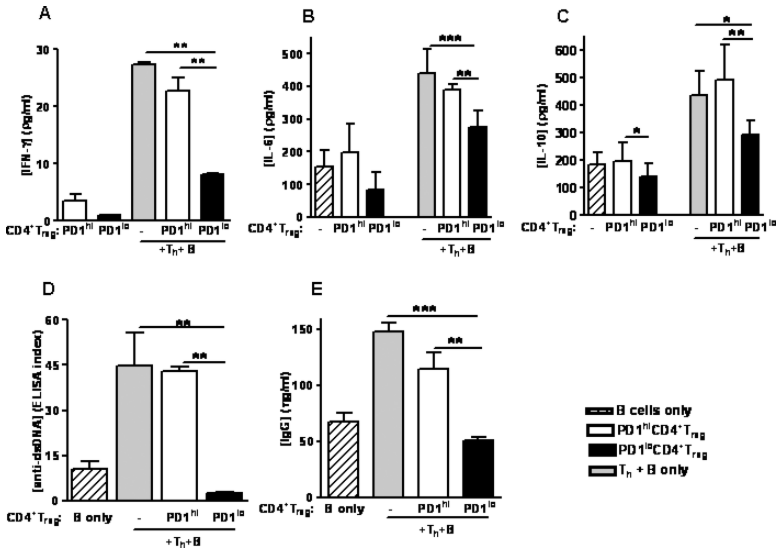

Fig 4. Reduction of proinflammatory cytokine and autoAb production in co-cultures with syngeneic CD4$^+$CD25$^-$ T$_h$ and B cells

PD1^{lo}CD4$^+$T$_{reg}$ added to cultures were induced by adding anti-PD-1 Ab *in vitro* to CD4$^+$CD25$^+$ T cells from unmanipulated mice. CD4$^+$T$_{reg}$ from 22 week-old unmanipulated BWF$_1$ mice (PD1^{hi} CD4$^+$T$_{reg}$) were treated with anti-PD-1 Ab at 75 μg/ml for 24 hours to generate PD1^{lo}CD4$^+$T$_{reg}$, followed by washing with PBS, then cocultured for 48 hours with T$_h$ and B cells from unmanipulated syngeneic mice. PD1^{lo} CD4$^+$T$_{reg}$ decreased the production of (A) IFN-γ, (B) IL-6, (C) IL-10, (D) anti-DNA and (E) IgG (Fig. 4A-E, $p < 0.001$, one-way ANOVA). PD1^{hi}CD4$^+$T$_{reg}$ and B cells alone from these mice were used as controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Tukey's test). Data are representative of 3 individual experiments, n = 5 mice per group in each experiment.

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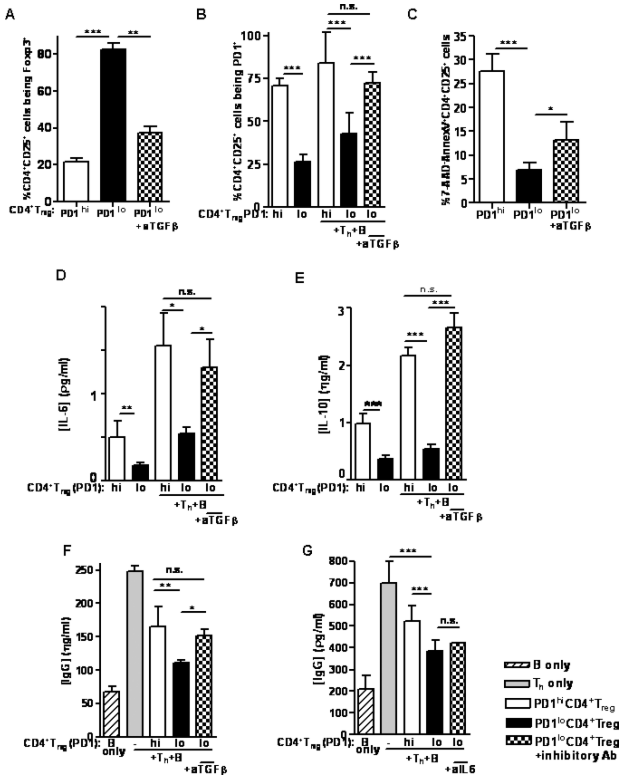

Fig 5. TGF-β was necessary to maintain the suppressive capacity of PD1^{lo}CD4^{+}T$_{reg}$ generated by *in vitro* treatment of CD4^{+}CD25^{+} T cells

CD4^{+}T$_{reg}$ from 22 week-old unmanipulated BWF$_1$ mice (PD1^{hi}CD4^{+}T$_{reg}$) were treated *in vitro* with anti-PD-1 Ab at 75 μg/ml for 24 hours (generating PD1^{lo}CD4^{+}T$_{reg}$), then washed, cultured with neutralizing anti-TGF-β mAb (or control mAb) at 5 mg/ml for 12 hours, then washed, and finally cocultured for 48 hours with syngeneic T$_h$ and B cells from unmanipulated mice. The regulatory function attained in PD1^{lo}CD4^{+}T$_{reg}$ was abrogated when these CD4^{+}T$_{reg}$ were pretreated with anti-TGF-β. Anti-TGF-β (A) decreased Foxp3 expression, (B) increased PD-1 expression and (C) increased apoptosis in CD4^{+}T$_{reg}$ (Fig. 5 A-C, $p < 0.0001$, one-way ANOVA). In the supernatant of the coculture, there was increased production of (D) IL-6, (E) IL-10, and (F) IgG (Fig. 5D, 5F, $p < 0.001$; Fig. 5E, $p < 0.0001$, one-way ANOVA). (G) The role of IL-6 in the suppressive function of PD1^{lo}CD4^{+}T$_{reg}$ was tested by repeating the same setup, but treating PD1^{lo}CD4^{+}T$_{reg}$ with an inhibitory IL-6 Ab *in vitro*. The ability to suppress IgG production was unaffected in these PD1^{lo}CD4^{+}T$_{reg}$ (n.s., one-way ANOVA). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Tukey's test). Data are representative of 2 individual experiments, n = 6 mice per group in each experiment.

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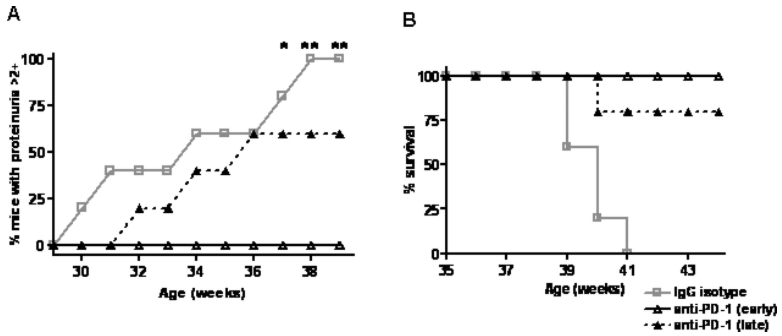

Fig 6. Comparison of clinical results of in vivo treatment of BWF_1 mice with PD-1 blockade before and after onset of autoAb production

BWF_1 mice at age 22 weeks (defined as "late" treatment in the legend) that had anti-dsDNA Ab, but proteinuria <2+ on urinary dipstick, were treated with 3 intraperitoneal injections of anti-PD-1 Ab at 100 μg per injection every other day. (A) Proteinuria over time in all treatment groups ("early" group was treated with anti-PD1 at 10 weeks of age). Onset of proteinuria was delayed in mice treated either early or late, although early treatment had a better effect. Controls were mice with late treatment with IgG matched to anti-PD1. ($p < 0.0001$, logrank test; anti-PD-1 (late) vs IgG isotype, anti-PD-1 (early) vs IgG isotype, anti-PD-1 (late) vs, anti-PD-1 (early), $p < 0.01$, Bonferroni method). (B) Mice with early and late treatment had prolonged survival when compared with the IgG isotype controls, ($p < 0.0001$, logrank test; anti-PD-1 (late) vs IgG isotype, anti-PD-1 (early) vs IgG isotype, $p < 0.001$, anti-PD-1 (late) vs anti-PD-1 (early), n.s., Bonferroni method). n = 20 mice per group in each experiment.