Depletion of PD-1-positive cells ameliorates autoimmune disease

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Targeted suppression of autoimmune diseases without collateral suppression of normal immunity remains an elusive yet clinically important goal. Targeted blockade of programmed-cell-death-protein-1 (PD-1)—an immune checkpoint factor expressed by activated T cells and B cells—is an efficacious therapy for potentiating immune activation against tumours. Here we show that an immunotoxin consisting of an anti-PD-1 single-chain variable fragment, an albumin-binding domain and *Pseudomonas exotoxin* targeting PD-1-expressing cells, selectively recognizes and induces the killing of the cells. Administration of the immunotoxin to mouse models of autoimmune diabetes delays disease onset, and its administration in mice paralysed by experimental autoimmune encephalomyelitis ameliorates symptoms. In all mouse models, the immunotoxin reduced the numbers of PD-1-expressing cells, of total T cells and of cells of an autoreactive T-cell clone found in inflamed organs, while maintaining active adaptive immunity, as evidenced by full-strength immune responses to vaccinations. The targeted depletion of PD-1-expressing cells contingent to the preservation of adaptive immunity might be effective in the treatment of a wide range of autoimmune diseases.

Autoimmune diseases are primarily mediated by autoreactive lymphocytes and/or their secreted autoantibodies1–4. Targeted suppression of certain lymphocyte populations is an effective strategy to treat these diseases, which has yielded new therapies for multiple sclerosis (MS) and systemic lupus erythematosus (SLE)5–8. However, these therapies are rarely considered as first-line therapeutic options due to their indiscriminate inhibition of normal adaptive immunity9–12. This inhibition occurs because these therapies target lymphocytes too broadly9–12. Thus, identification and selective suppression of pathogenic lymphocytes responsible for autoimmune diseases while keeping non-pathologic lymphocytes intact constitutes an overarching yet unmet clinical goal.

Cells expressing programmed-cell-death-protein-1 (PD-1) are primarily activated B and T cells or effector B and T cells13–15. PD-1, a negative receptor of these cells, switches on the PD-1 immune checkpoint when engaged by its ligands. This critical checkpoint counteracts immune-stimulatory signals and limits PD-1+ effector cells from initiating autoimmune destruction16–22. However, in type 1 diabetes (T1D), MS, SLE, and rheumatoid arthritis, the PD-1 checkpoint fails to stop autoimmune destruction16,21. Instead, PD-1+ cells infiltrate tissues17–19, and this infiltration escalates as the autoimmune diseases progress20. These observations indicate that PD-1+ cells are important mediators of autoimmune diseases. Consistent with this concept, the blockade of the PD-1 checkpoint, which leads to a proliferation of PD-1+ cells, exacerbates autoimmune diseases in both human and mouse models17,18–20. Taken together, the targeted depletion of PD-1+ cells in the context of autoimmune diseases might be an effective method to assuage autoimmunity. It is worth mentioning that PD-1+ cell depletion is a very different concept to ablation of the PD-1 gene. PD-1+ cell depletion eliminates activated lymphocytes; by contrast, the knockout of the PD-1 gene leaves activated lymphocytes uncontrolled by the PD-1 checkpoint and enables uncontrolled proliferation of activated lymphocytes21. Thus, whereas PD-1+ knockout predisposes the host to enhanced autoimmunity, we hypothesize that PD-1+ cell depletion suppresses autoimmunity.

There are two intrinsic advantages to using PD-1+ cell depletion. First, the depletion should leave naive (PD-1–) lymphocytes intact, and hence preserve B- and T-cell repositories because the depletion primarily applies to activated lymphocytes. PD-1+ cell depletion should not substantially compromise normal adaptive immunity. This distinguishes PD-1+ cell depletion from drugs currently used to treat autoimmune diseases, such as natalizumab and alemtuzumab14,15. Second, PD-1+ cell depletion applies to both activated B cells and activated T cells, since both cell types are PD-1+. The dual coverage of both activated B and T cells is advantageous because the both cell types can contribute to autoimmune diseases1.

Here, we describe a fusion protein, containing a single-chain variable fragment (scFv) of the PD-1 antibody (αPD-1)23,24, an albumin-binding domain (ABD)25,26, and a *Pseudomonas aeruginosa* exotoxin A (PE)27,28, as a tool for PD-1+ cell depletion. In the resulting immunotoxin (αPD-1–ABD–PE), αPD-1 serves as a targeting moiety. ABD is used to extend plasma presence, because ABD-containing molecules have a long half-life in plasma20,29, whereas PE has been demonstrated to have clinical efficacy and is safe30,31. αPD-1–ABD–PE possesses selective toxicity, both in vitro and in vivo, to PD-1+ cells. More importantly, αPD-1–ABD–PE not only halted the progression of autoimmune diseases in our systems, but also concomitantly preserved normal adaptive immunity.

Results

αPD-1–ABD–PE selectively binds and penetrates PD-1+ cells. αPD-1–ABD–PE has three functional components: αPD-1 (scFv),...
ABD and PE (Fig. 1a). The amino acid sequences of ABD and PE have been previously published31,34. We sequenced RMP1–14, a rat monoclonal antibody raised against mouse PD-1 (IgG2a, κ)35. On the basis of these sequencing results, we designed αPD-1 to contain two mutations, V38 (R45C) and V91 (G104C) (Fig. 1b). The two cysteines were introduced to form a disulfide bond and enhance stability of αPD-114. A linker, (GGGGS)3, was used to connect αPD-1, ABD, and PE. We also designed three control proteins for this study: αPD-1, ABD–PE, and αPD-1–PE (Fig. 1a). All of these proteins have a histidine tag (HisTag) to facilitate purification. The coding genes for αPD-1–ABD–PE and the control proteins were cloned into the pET25b(+) vector. The sizes of the cloned genes were confirmed by agarose gel electrophoresis (Supplementary Fig. 1a), and the genes were sequenced and confirmed to have the designed sequences. αPD-1–ABD–PE and the control proteins were produced and purified as soluble proteins from Escherichia coli (Shuffle T7) that harboured the expression vectors. The yield was approximately 0.3 mg/l culture for each of the proteins. The purity and size of these proteins were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Supplementary Fig. 1b). The sizes of these proteins were consistent with their predicted theoretical molecular masses; for example, αPD-1–ABD–PE migrated slightly lower than the 63 kDa marker, in agreement with its theoretical molecular mass of 57.9 kDa. Endotoxins were removed from all purified proteins to 0.1 U per mg protein.

In comparisons between PD-1+ and PD-1− cells of different types, we found that αPD-1–ABD–PE selectively binds to PD-1+ cells. We compared PD-1+ and PD-1− primary T cells, PD-1+ and PD-1− primary B cells, and EL4 (PD-1+)]36 and B16-F10 (hereafter referred to as B16) (PD-1−) cells (Supplementary Fig. 2a,b). We incubated Alexa Fluor 647-labelled αPD-1–ABD–PE with PD-1+ and PD-1− cells, separately at 4 °C (30 min), whereby cell-surface binding could occur but internalization was inhibited. After incubation, the mean fluorescence intensity (MFI) of the PD-1+ primary T cells was more than four times that of the PD-1− primary T cells (188.33 ± 5.92 versus 43.45 ± 2.98; Fig. 1c); the MFI of the PD-1+ primary B cells was more than five times that of the PD-1− primary B cells (229.67 ± 12.45 versus 43.72 ± 2.46; Fig. 1d); and the MFI of EL4 cells was more than three times that of the B16 cells (571.70 ± 12.12 versus 116.00 ± 1.61; Supplementary Fig. 2c). By contrast, both the PD-1+ and the PD-1− cells had low MFI after similar incubations with labelled ABD–PE (Fig. 1c,d and Supplementary Fig. 2c). Using a dose-responsive binding assay, we found that the apparent dissociation constant (Kd) of αPD-1–ABD–PE on PD-1+ EL4 cells is 3.4 nM (95% CI 2.2–5.2 nM; Supplementary Fig. 2d), indicating that binding and internalization could progress. After incubation, the mean fluorescence intensity (MFI) of the PD-1+ primary T cells (74.52 ± 0.63 versus 1895.00 ± 34.82, P < 0.0001; Fig. 1g); and at 37 °C, the MFI resulting from the concurrent incubation was approximately 1/25 that from the single incubation (74.52 ± 0.63 versus 1895.00 ± 34.82, P < 0.0001; Fig. 1g). These data corroborate our finding that the binding and internalization of αPD-1–ABD–PE into PD-1+ cells were directed by PD-1−. Interestingly, when EL4 cells were pre-incubated with αPD-1–ABD–PE at 37 °C for 30 min before PD-L1–Fc was added to the mixture, PD-L1–Fc only partially decreased the MFI of the EL4 cells, from 1895.00 ± 34.82 to 1320.00 ± 120.30 (Fig. 1g). The partial decrease may be because the PD-L1–Fc displaced surface-bound αPD-1–ABD–PE, but some αPD-1–ABD–PE had entered the EL4 cells before the addition of PD-L1–Fc and, consequently, could not be displaced by PD-L1–Fc. This observation provides evidence that αPD-1–ABD–PE enters PD-1+ cells. This conclusion regarding PD-1-mediated internalization is likely to hold for PD-1+ cells of different types and origins, since the results are consistent between PD-1+ primary T and B cells, and between cells collected from C57BL/6 and NOD mice (Supplementary Fig. 3).

αPD-1–ABD–PE specifically depletes PD-1+ cells both in vitro and in vivo. In vitro, αPD-1–ABD–PE was found to be at least 100 times more cytotoxic to PD-1+ primary lymphocytes than to PD-1− primary lymphocytes: the half-maximal inhibitory concentration (IC50) of αPD-1–ABD–PE for PD-1+ primary T cells was 0.43 nM (95% CI = 0.270–0.69 nM), whereas αPD-1–ABD–PE did not show detectable cytotoxicity towards PD-1− primary T cells up to 100 nM (Fig. 2a); the IC50 of αPD-1–ABD–PE for PD-1+ primary B cells was 0.47 nM (95% CI = 0.34–0.63 nM), whereas αPD-1–ABD–PE did not show toxicity towards PD-1− primary B cells up to 100 nM (Fig. 2b). A control mixture of αPD-1–ABD–PE did not show cytotoxicity to either PD-1+ or PD-1− primary lymphocytes up to 100 nM (Fig. 2a,b). The large difference in cytotoxicities of αPD-1–ABD–PE and the control mixture indicated that the cytotoxicity of αPD-1–ABD–PE is dependent on a contiguous linkage between αPD-1 and ABD–PE. Similar results were observed with primary lymphocytes from both C57BL/6 (Fig. 2a,b) and NOD mice (Supplementary Fig. 4a,b).

To pinpoint the role of αPD-1 in the selective cytotoxicity of αPD-1–ABD–PE to PD-1+ cells, we generated a PD-1 knockout EL4 cell line (PD-1− EL4) using clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR associated protein 9 (Cas9) (Supplementary Fig. 4c). We found that αPD-1–ABD–PE was more than 1,000 times more toxic to wild-type EL4 cells than to PD-1− EL4 cells (IC50 = 0.64 nM, 95% CI = 0.49–0.83 nM versus IC50 = 1,120 nM, 95% CI = 702.7–1,773.0 nM; Fig. 2c). By contrast, a control mixture of αPD-1 and ABD–PE showed little or no cytotoxicity towards both cell lines (IC50 = 590 nM, 95% CI = 458.7–760.6 nM for wild-type EL4 cells; IC50 = 1,060 nM, 95% CI = 747.7–1,514.0 nM for PD-1− EL4 cells). Together, these results point to a clear role of PD-1 in the PD-1− cell-selective toxicity of αPD-1–ABD–PE.
**Fig. 1 | αPD-1-ABD-PE specifically binds to and enters PD-1⁺ lymphocytes.**

αPD-1-ABD-PE was also found to effectively deplete PD-1⁺ cells in vivo. Specifically, a single dose of αPD-1-ABD-PE reduced the fraction of adoptively transferred EL4 cells among circulating lymphocytes in hosts to 2.86 ± 0.46%, whereas mice treated with the mixture of αPD-1 and ABD-PE had an unchanged EL4 cell fraction compared with PBS-treated mice (14.93 ± 1.36% versus 14.37 ± 1.60%; Fig. 2d). The radically different cell depletion results between αPD-1-ABD-PE and the control mixture again confirm the importance of the linkage between αPD-1 and ABD-PE.
ABD increases the plasma exposure of αPD-1–ABD–PE. We also examined the function of ABD in αPD-1–ABD–PE. Native PAGE analysis showed a clear association of αPD-1–ABD–PE with mouse serum albumin (MSA, Fig. 3a) and human serum albumin (HSA, Fig. 3b). By contrast, αPD-1–PE did not associate with either MSA or HSA. These data suggest that the ABD component of αPD-1–ABD–PE retains its albumin-binding capacity, and that the interaction between αPD-1–ABD–PE and albumin are dependent on the presence of the ABD. Next, we examined the pharmacokinetics of αPD-1–ABD–PE and αPD-1–PE after intraperitoneal injection of 5.0 nmol of protein per mouse (Fig. 3c). A non-compartmental analysis shows that αPD-1–ABD–PE has a systemic clearance (CL) approximately 1/30 that of αPD-1–PE (76.35 ± 9.13 h⁻¹ versus 1.23 ± 0.01 h⁻¹), resulting in 30 times higher plasma exposure (area under curve, AUC₀–∞) of αPD-1–ABD–PE (35.63 ± 2.31 μM h) as compared with αPD-1–PE (0.14 ± 0.01 μM h). These data suggest that the construction of αPD-1–ABD–PE improves the pharmacokinetics of the construct, which was expected to benefit the efficacy of αPD-1–ABD–PE, since poor pharmacokinetics are reported to limit the efficacy of PD-1–ABD–PE improved the pharmacokinetics of the construct, versus the mixture control). These data, together with the spontane-
ment would diminish PD-1⁺ in NOD mice25,39,41, we investigated whether αPD-1–ABD–PE treatment significantly reduced the fraction of PD-1⁺ cells in pancreas as compared with PBS (0.17 ± 0.02% versus 0.39 ± 0.03%, P=0.0002; Fig. 4c). By contrast, the control mixture did not reduce the fraction of PD-1⁺ cells in the pancreas (0.56 ± 0.07%, P=0.052). The level of PD-1 expression (MFI) in PD-1⁺ cells that survived the αPD-1–ABD–PE treatment was slightly but significantly lower (2.132.17 ± 16.82, P=0.025) than in the PD-1⁺ surviving the PBS treatment (2.187.00 ± 12.12, Supplementary Fig. 6a). There was no significant difference in PD-1 expression between the cells from the PBS and the control mixture treatments. These results may be due to preferential targeting of αPD-1–ABD–PE to PD-1⁺ cells over PD-1⁻ cells. Further, αPD-1–ABD–PE treatment also significantly reduced the fractions of PD-1⁺ CD4 T cells and PD-1⁺ CD8 T cells in the pancreas as compared with PBS (P=0.0007 and 0.0002, respectively; Fig. 4d,e). By contrast, the control mixture did not reduce the fractions of these two populations of PD-1⁺ lymphocytes, and slightly increased the PD-1⁺ CD4 T-cell fraction. These results also revealed that PD-1⁺ CD4 T cells are the major PD-1⁺ cells in the pancreases of the PBS-treated mice, accounting for approximately 75% of the PD-1⁺ cells. There was no detectable population of PD-1⁺ Tₘₜ cells or PD-1⁺ B cells in the pancreases (Supplementary Fig. 6b,c). The αPD-1–ABD–PE treatment also reduced total CD4 and CD8 cells but not B cells in the pancreases, as compared with PBS (P=0.0002, 0.0008 and 0.119, respectively; Fig. 4f–h). The control mixture, as expected, did not affect these cells. Thus, αPD-1–ABD–PE reduced the fractions of total PD-1⁺ cells, PD-1⁺ CD4 T cells, PD-1⁺ CD8 T cells, CD4 T cells, and CD8 T cells in the pancreases of treated mice, which might contribute to its marked delaying effect on the onset of T1D. Among these populations, PD-1⁺ CD4 T cells are likely to be a decisive factor, given that they represent a major fraction of the PD-1⁺ cells (75%) and their numbers were reduced by approximately 3 times after the αPD-1–ABD–PE treatment. We also examined the PD-1⁺ T cells and PD-1⁺ B cells in blood, spleens, and lymph nodes of these treated mice. However, these cells did not form distinguishable populations, probably owing to their scarcity (Supplementary Fig. 6d–i). In other words, the baseline numbers of PD-1⁺ cells in blood and peripheral lymphatic organs are very low. Because of this result, we do not expect the total T cell and B cell numbers in these organs to be altered by αPD-1–ABD–PE treatments.

In addition to the pancreatic infiltration of lymphocytes, there were other data suggesting that αPD-1–ABD–PE selectively depletes autoreactive PD-1⁺ cells in NOD mice (Fig. 4i). PD-1 antibody usually accelerates T1D progression in NOD mice because it allows autoreactive PD-1⁺ cells to proliferate[14,24-27]. In this study, αPD-1 accelerated the onset of T1D in NOD mice that were pretreated with PBS and the mixture control: the median T1D-free survivals were 15 and 13 days, respectively (Fig. 4i). By contrast, the T1D exacerbating effect of αPD-1 diminished in NOD mice pre-treated with αPD-1–ABD–PE; these mice did not show hyperglycaemia even at the end of the experiment (26 days after the treatment). Because autoreactive PD-1⁺ cells are the primary effector cells that cause T1D and execute the exacerbating effect of αPD-1, the diminished effect of αPD-1 in the mice pre-treated with αPD-1–ABD–PE suggests that there is a reduction of autoreactive PD-1⁺ cells in these mice.

αPD-1–ABD–PE treated mice recover from EAE. C57BL/6 mice immunized with a peptide of myelin oligodendrocyte glycoprotein (MOG₅₅₋₅₉) in adjuvant develop experimental autoimmune encephalomyelitis (EAE), a monophasic clinical disease from which mice rarely recover16,41. To test whether αPD-1–ABD–PE modulates EAE, C57BL/6 mice with EAE were treated with either: (1) αPD-1–ABD–PE, (2) PBS, or (3) a mixture of αPD-1 and ABD–PE. Before the treatments, all the mice had paralysed hind limbs, a sign of severe EAE (clinical score 3.0). All six of the mice treated with αPD-1–ABD–PE recovered from the paralytic diseases after a single dose

### Table 1 | Summary of key pharmacokinetic parameters derived from non-compartmental analysis

| Sample    | αPD-1–ABD–PE | αPD-1–PE |
|-----------|--------------|----------|
| CL (ml h⁻¹) | (mean ± s.d.) | (mean ± s.d.) |
| 0.14 ± 0.01 | 4.05 ± 0.02 |
| AUC₀–∞ (μM h) | 35.63 ± 2.31 | 1.23 ± 0.01 |
| t½ (h) | 76.35 ± 9.13 | 1.34 ± 0.02 |
| Vₐ (ml) | 15.43 ± 1.06 | 7.82 ± 0.15 |
Fig. 2 | αPD-1–ABD–PE is selectively toxic to PD-1+ cells in vitro and in vivo. a. The relative viability of PD-1+ and PD-1− primary T cells from C57BL/6 mice after the cells were incubated with αPD-1–ABD–PE or a control mixture of αPD-1 and ABP–PE for 72 h. Viabilities are shown as mean ± s.d. at different concentrations of αPD-1–ABD–PE and control mixture. The viability data for PD-1+ primary T cells with αPD-1–ABD–PE treatment are fitted to a sigmoidal dose-response model and the IC50 was obtained (n = 6 biologically independent samples). b. The relative viability of PD-1+ and PD-1− primary B cells from C57BL/6 mice after the cells were incubated with αPD-1–ABD–PE or a control mixture of αPD-1 and ABP–PE for 72 h. Viabilities are shown as mean ± s.d. at different concentrations of αPD-1–ABD–PE and control mixture (n = 6 biologically independent samples). c. The relative viability of wild-type EL4 and PD-1–EL4 cells after they were incubated with αPD-1–ABD–PE or a control mixture of αPD-1 and ABP–PE for 72 h. Viabilities are shown as mean ± s.d. at different concentrations of αPD-1–ABD–PE and control mixture fitted to a sigmoidal dose-response model (n = 6 biologically independent samples). d. The fractions of transferred EL4 cells among lymphocytes collected from mice 72 h after the mice were treated with αPD-1–ABD–PE, a control mixture of αPD-1 and ABP–PE, or PBS. Data are mean ± s.d. (n = 3 mice). All studies described in this figure were repeated twice with similar results. Data from one repeat are shown here.

(Fig. 5a,b), but all the mice had an EAE clinical score of 1.0 at the end of the study. In another replicate study, two of five mice treated with αPD-1–ABD–PE showed no clinical sign of EAE (fully recovered) at the end of the study (clinical score 0, Supplementary Fig. 7a), whereas the other three mice had clinical scores of 1.0. By contrast, none of the paralysed mice that received PBS or the control mixture treatment recovered. Instead, their EAE steadily worsened. The EAE scores of all mice in these two groups reached 4.0 by the end of this study. Indeed, from day 5 after treatment, the mean clinical score of the αPD-1–ABD–PE treatment group was consistently and significantly lower than the scores of the PBS- and the control mixture-treated groups. It is important to note that mice in the three groups developed EAE with the same kinetics before the various treatments were applied (Fig. 5a): there was no significant difference in mean EAE scores among the three groups until day 19 after EAE induction (P > 0.05 for all the monitored time points). At that time, some mice had received treatments, and the treatment had started to alter their EAE progression. The identical kinetics of EAE development among the three groups before the treatments, conversely, underscores the power of αPD-1–ABD–PE to interfere with and reverse the disease progression. We investigated whether αPD-1–ABD–PE treatment reduced the infiltrations of PD-1+ cells and lymphocytes into the central nervous system (CNS) of the mice with EAE. The presence of these cells, especially CD4 cells, in the CNS has been linked to EAE progression. Even a single dose of αPD-1–ABD–PE reduced the fraction of PD-1+ cells in the CNS to 20% of the group treated with PBS (Fig. 5c; 0.19 ± 0.03 % versus 1.07 ± 0.09 %, P < 0.0001). By contrast, the control mixture of αPD-1 and ABP–PE did not reduce the fraction of PD-1+ cells in the CNS (1.19 ± 0.07 %, P = 0.341). PD-1+ cells collected from the αPD-1–ABD–PE-treated mice also exhibited significantly lower PD-1 expression (1.793.83 ± 11.55, P = 0.0013).
We also examined, but did not detect, PD-1⁺ cells and PD-1⁺ B cells in blood, spleen and lymph nodes of treated EAE mice (Supplementary Fig. 7g–l).

αPD-1–ABD–PE does not compromise normal adaptive immune responses. The αPD-1–ABD–PE treatment did not cause lymphopenia, which is often associated with therapeutics for autoimmune diseases. We compared the B220⁺, CD4⁺ and CD8⁺ lymphocytes in blood and spleens of C57BL/6 mice (Fig. 6a) after they received one dose of: (1) αPD-1–ABD–PE, (2) PBS, (3) a mixture of αPD-1 and ABD–PE, or (4) cyclophosphamide (CP). CP is a non-specific immunosuppressant used here as a positive control for immune suppression. αPD-1–ABD–PE did not reduce the numbers of these three lymphocyte subpopulations in comparison to the PBS treatment (Fig. 6a,b). Similarly, the mixture control did not affect the proportions of these lymphocyte subpopulations. However, cyclophosphamide reduced the numbers of B220⁺, CD4⁺ and CD8⁺ lymphocytes in blood by 70.1%, 69.5% and 75.6%, respectively (P < 0.0001, P = 0.0002 and P = 0.0002, respectively; Fig. 6a). CP reduced the numbers of B220⁺, CD4⁺ and CD8⁺ lymphocytes in spleens by 46.0%, 76.0% and 70.1%, respectively (P < 0.0001, P = 0.0002, P < 0.0001 and P < 0.0001, respectively; Fig. 6b). These findings were reproducible in NOD mice (Supplementary Fig. 8a,b).

The αPD-1–ABD–PE treatment did not affect antibody responses in treated mice. We treated C57BL/6 and NOD mice with one dose of: (1) αPD-1–ABD–PE, (2) PBS, (3) a mixture of αPD-1 and ABD–PE, or (4) CP. Two days after this treatment, the mice were immunized with 2,4-dinitrophenyl-Ficoll (DNP–Ficoll), a T cell-independent antigen. We found that both the

Fig. 3 | αPD-1–ABD–PE binds to albumin and has enhanced plasma exposure. a, Native PAGE analysis, demonstrating the association between αPD-1–ABD–PE and MSA. αPD-1–ABD–PE and MSA were mixed in a 1:1 ratio. αPD-1–PE and MSA were also mixed in a 1:1 ratio. b, Native PAGE analysis, demonstrating the association between αPD-1–ABD–PE and HSA. αPD-1–ABD–PE and HSA were mixed in a 1:1 ratio. αPD-1–PE and HSA were also mixed in a 1:1 ratio. c, Plasma concentration versus time profiles of αPD-1–ABD–PE and αPD-1–PE after the two proteins were intraperitoneally injected into mice at 5 nmol per mouse. Data are mean + s.d. (n = 3 biologically independent samples). The pharmacokinetic data was analysed using a non-compartmental model. Each dot represents a plasma concentration value at a given time point.
Fig. 4 | Administration of αPD-1–ABD–PE delays the onset of TID. a, Diabetes-free survival of NOD mice treated weekly with αPD-1–ABD–PE, a control mixture of αPD-1 and ABD–PE, or PBS from 12 weeks of age (n = 5 mice). b, Diabetes-free survival of NOD mice treated five times with αPD-1–ABD–PE, a control mixture of αPD-1 and ABD–PE, or PBS (n = 5 mice). c, The fraction of PD-1+ CD4 T cells in one mouse. Fractions are shown as mean ± s.d. (n = 6 mice; unpaired two-sided t-test). d, The fraction of PD-1+ CD4 T cells among the pancreatic cells described in c. Each dot represents the fraction of PD-1+ CD4 T cells in one mouse. Fractions are shown as mean ± s.d. (n = 6 mice; unpaired two-sided t-test). e, The fraction of PD-1+ CD8 T cells among the pancreatic cells described in c. Each dot represents the fraction of PD-1+ CD8 T cells in one mouse. Fractions are shown as mean ± s.d. (n = 6 mice; unpaired two-sided t-test). f, The fraction of CD4 T cells among the pancreatic cells described in c. Each dot represents the fraction of CD4 T cells in one mouse. Fractions are shown as mean ± s.d. (n = 6 mice; unpaired two-sided t-test). g, The fraction of CD8 T cells among the pancreatic cells described in c. Each dot represents the fraction of CD8 T cells in one mouse. Fractions are shown as mean ± s.d. (n = 6 mice; unpaired two-sided t-test). h, The fraction of B cells among the pancreatic cells described in c. Each dot represents the fraction of B cells in one mouse. Fractions are shown as mean ± s.d. (n = 6 mice; unpaired two-sided t-test). i, Diabetes-free survival of NOD mice treated first with αPD-1–ABD–PE, a control mixture of αPD-1 and ABD–PE, or PBS, and then with αPD-1 (full IgG) (n = 5 mice; unpaired two-sided t-test) The survival of mice treated with αPD-1–ABD–PE is significantly different to that of mice treated with PBS or the control mixture treated (P = 0.0494 or P = 0.0018, respectively). All studies described in this figure were repeated twice with similar results. Data from one repeat are shown here.

αPD-1–ABD–PE–treated mice and the control mixture–treated mice developed the same level of anti-DNP responses as the PBS–treated mice (Fig. 6b and Supplementary Fig. 8c–f). However, CP–treated mice developed weaker anti-DNP responses. These findings were reproduced in both C57BL/6 and NOD mice. Of note, similar results were observed in mice that were treated with five doses of each treatment (Supplementary Fig. 8g,h).

The αPD-1–ABD–PE treatment did not affect cytotoxic T lymphocyte (CTL) responses. We treated C57BL/6 and NOD mice with one dose of: (1) αPD-1–ABD–PE, (2) PBS, (3) a mixture of αPD-1 and ABD–PE, or (4) CP. Two days after this treatment, NOD mice were immunized with TYPQRAIL, a CTL-epitope vaccine matched to the major histocompatibility complex (MHC) class I background of NOD mice. At the same time, treated C57BL/6 mice were immunized with SIINFEKL, a CTL vaccine that was derived from ovalbumin (residues 257–264) and matched with the MHC class I background of C57BL/6 mice. Mice treated with αPD-1–ABD–PE, control mixture or PBS all developed similar levels of
CTL responses (Fig. 6c). By contrast, C57BL/6 and NOD mice treated with CP developed weaker CTL responses as compared with those treated with PBS ($P = 0.011$ and $P = 0.026$ for C57BL/6 and NOD mice, respectively).

Together, these data show that αPD-1–ABD–PE treatment did not significantly alter the ability of the treated mice to mount antibody- and/or CTL responses.

**Discussion**

The results of this study show that αPD-1–ABD–PE exhibits specific toxicity towards PD-1+ B and PD-1+ T cells. Depletion of PD-1+ cells, enabled by αPD-1–ABD–PE, ameliorates autoimmunity in multiple disease models that are different in pathogenesis. Furthermore, application of PD-1+ cell depletion does not cause adaptive immune deficiency.
In a chronic EAE model, even a single dose of αPD-1–ABD–PE fully restored mobility in mice that were paralysed by EAE, a rarely achieved therapeutic outcome. In addition, the single dose of αPD-1–ABD–PE significantly reduced numbers of PD-1+ cells, T lymphocytes and autoreactive lymphocytes in the CNS of the treated mice. Current therapies for MS are termed disease-modifying therapies because they only delay disease progression and do not reverse or cure (or cure) the disease[6,11]. The noted efficacy of PD-1+ cell depletion may be able to fill an important clinical gap in MS treatment. In a spontaneous model of T1D and in two accelerated-T1D models (CP treatment and αPD-1 IgG treatment), PD-1+ cell depletion markedly delayed T1D onset. Under the studied conditions, T1D eventually developed in the spontaneous and the CP-accelerated models. Nevertheless, it is possible that the efficacy of PD-1+ cell depletion could be further boosted by regimens that are optimized for the pharmacokinetics–pharmacodynamics of αPD-1–ABD–PE. The improved efficacy may allow the depletion to completely prevent T1D. This possibility is supported by the observed broad impact of a single dose of αPD-1–ABD–PE on immune cells in pancreases; the single dose decreases the fractions of PD-1+ CD4 T cells and PD-1+ CD8 T cells, as well as total CD4 and CD8 T cells, a global attenuation of the inflammation in pancreases. PD-1+ cell depletion may also help to reverse T1D in combination with β-cell compensation therapy[24]. It is notable that PD-1+ cell depletion delayed the onset of CP-induced T1D. This observation implies first that PD-1+ cells may be the primary effector cells in autoimmunity driven by Treg deficiency, since CP causes Treg deficiency[30]. Second, it suggests that PD1+ cell depletion may be able to resolve the autoimmunity that does not originate from the abnormality of the PD-1 checkpoint. Together, PD-1+ cell depletion is effective against EAE and T1D, two T cell-driven autoimmune disease models; PD-1+ CD4 T cells appeared to be the major pathogenic cell population in the two models, and both responded well to PD-1+ cell depletion.

PD-1+ cell depletion is likely to be effective against B cell-mediated autoimmune diseases. First, such autoimmune diseases, like SLE, depend on not only B cells but also CD4 T cells such as follicular helper T (Tfh) cells and extrafollicular helper T (Teff) cells. These CD4 T cells facilitate the formation of germinal centre, the maturation of B cells, and antibody production[44]. Our results also show that PD-1+ cell depletion diminished both PD-1+ CD4 T cells and total CD4 T cells (Figs. 4 and 5). Further, our results confirm

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**Fig. 6** Administration of αPD-1–ABD–PE does not affect normal adaptive immune responses. a. B220+, CD4+, CD8+ cell numbers in blood and spleens of C57BL/6 mice treated with one dose of αPD-1–ABD–PE, a control mixture of αPD-1 and ABD–PE, PBS, or cyclophosphamide (CP). Data are mean ± s.d. (n = 6 mice; unpaired two-sided t-test). b. ELISA results showing anti-DNP humoral responses in C57BL/6 and NOD mice pre-treated with one dose of αPD-1–ABD–PE, a control mixture of αPD-1 and ABD–PE, PBS, or CP. The results were measured as A0.570 nm, subtracting background A0.570 nm. Data are mean ± s.d. for the serum samples at the indicated dilutions. The same samples were loaded into both DNP–BSA-coated and BSA-coated (control) ELISA plates, separately. The materials used to coat the plates are shown in parentheses. n = 6 mice. c. ELISpot assay results showing CTL responses in C57BL/6 and NOD mice pre-treated with one dose of αPD-1–ABD–PE, a control mixture of αPD-1 and ABD–PE, PBS, or CP. Data represent mean ± s.d. of the number of IFN-γ-positive spots resulting from 500,000 splenocytes collected from the treated mice (n = 6 mice; unpaired two-sided t-test; C57BL/6 mice: NS, not significant, P = 0.9429). All studies described in this figure were repeated at least twice with similar results. Data from one repeat are shown here.
that αPD-1–ABD–PE is able to specifically bind to, penetrate, and eliminate PD-1+ primary B cells. Finally, PD-1 knockout mice show increased IgG production and eventually develop lupus-like symptoms. One interpretation for this observation is that activated autoreactive cells in the mice escape the PD-1 immune checkpoint and cause the lupus-like symptoms, since these cells do not express PD-1. By contrast, activated autoreactive cells are PD-1+ in wild-type mice, and hence inhibited by the checkpoint. In mice with SLE, the checkpoint fails to suppress activated autoreactive cells. However, it is possible to utilize PD-1 as a biomarker to identify and deplete the cells and rescue autoimmune destruction in the mice with SLE. In sum, PD-1+ cell depletion impacts both PD-1+ B cells and PD-1+ CD4+T cells and is likely to alleviate B-cell mediated autoimmune diseases.

PD-1+ cell depletion preserves normal adaptive immunity, which sharply contrasts with the generalized immune deficiency caused by currently available immune suppressants. It is acknowledged that PD-1+ cell depletion could diminish non-autoreactive PD-1+ lymphocytes, including PD-1+ effector cells and Treg cells. However, we feel that this represents only a theoretical concern. First, PD-1+ effector cells can be replenished from naive lymphocytes upon immune stimulation, as lymphocyte repositories are not impaired by PD-1+ cell depletion. Second, PD-1+ cells are scarce and barely detectable in blood and peripheral lymphatic organs in mice with T1D and EAE—and they are relatively concentrated in inflamed organs (Figs. 4 and 5 and Supplementary Figs. 6–7). This intrinsic distribution of PD-1+ cells helps to alleviate the negative impact of PD-1+ cell depletion on non-autoreactive lymphocytes. Third, the finding that PD-1+ cell depletion preserves adaptive immunity argues against the theoretical concern; mice in which PD-1+ cells were depleted were able to mount normal immune responses as soon as two days after the depletion. There are further arguments supporting the depletion of PD-1+ cells in autoimmune diseases. In the context of autoimmune diseases, the immune system is, overall, tilted towards autoimmunity rather than immune tolerance or energy. Autoreactive PD-1+ lymphocytes outweigh their non-autoreactive counterparts, including PD-1− Treg cells in terms of impact to the well-being of patients. Therefore, it is arguably beneficial to purge all PD-1+ cells, reprogramme the immune system, and restore immune homeostasis. PD-1+ cell depletion, indeed, resembles systemic γ-irradiation or lymphocyte depletion, which have both been used to reprogramme the immune system. Yet, PD-1+ cell depletion is a much more focused approach. One observation that supports this idea of purging PD-1+ cells is that the αPD-1–ABD–PE treatment, while reducing PD-1− CD4+ T cells, PD-1− CD8+ T cells, and autoreactive T cells in pancreases of NOD mice and the CNS of mice with EAE, did not diminish Treg cells in these organs. Indeed, the depletion increased the ratios of Treg cells to autoreactive cells in the CNS, which favours the treatment of autoimmune diseases.

Targeted depletion of PD-1+ cells, enabled by αPD-1–ABD–PE, exhibits three appealing features as a new therapeutic option for autoimmune diseases. First, the depletion has a straightforward and robust mechanism. It relies on a simple cytotoxic mechanism to kill PD-1+ cells; yet, a single administration can suppress autoimmunity completely, enabling, for example, paralysed mice to regain normal gait. Second, the depletion can suppress both T cell- and B cell-mediated autoimmunity in an autoimmune disease. It also has potential to treat autoimmune diseases driven primarily by T cells or B cells. Third, the PD-1− cell depletion does not cause long-term immune deficiency. Although the depletion can also affect non-autoreactive PD-1+ cells and may affect the healthy immunity during the treatment, the immunity appears to recover very quickly because of preservation of naïve cells. Indeed, as soon as two days after the depletion, αPD-1–ABD–PE-treated mice were able to mount the same level of adaptive responses as PBS-treated mice. Thus, PD-1+ cell depletion did not have a long-term impact on healthy immunity. In summary, targeted depletion of PD-1+ cells is an effective and broadly applicable approach to treating autoimmune diseases without jeopardizing healthy immunity.

Methods

Mice and cell lines. Female C57BL/6 mice were purchased from The Jackson Laboratory. Female NOD mice were purchased from The Jackson Laboratory and bred in-house. Animal studies were conducted following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah. The group sizes of mice were determined and approved by the regulatory authorities for animal welfare after a balanced consideration of statistical and scientific needs and ethical aspects. All procedures related to animal studies are in compliance with relevant ethical regulations on animal research at the University of Utah. EL4 and B16-F10 cell lines were purchased from ATCC and maintained in DMEM medium with 10% FBS.

Antibodies. Capture anti-mouse interferon-γ (IFN-γ) monoclonal antibody (clone R4–6A2) and biotinylated, detection anti-mouse IFN-γ monoclonal antibody (clone XMGL.2–biotin) were purchased from BioLegend. Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgM and Peroxidase AffiniPure Goat Anti-Mouse IgG (H + L) were purchased from Jackson ImmunoResearch. Anti-mouse CD3-α antibody (clone 145–2C11) was purchased from BioXCell. FITC-, PE-, APC- or biotin-conjugated monoclonal antibodies to Fsp23 (clone MF-14), B220 (clone RA3–6B2), CD4 (clone GK1.5), and CD8 (clone 53–67) were purchased from BioLegend.

Generation of expression vectors for recombinant proteins. Genes encoding αPD-1 (scFv), PE, or ABD–PE were synthesized by Biomatik. All the genes were inserted into the pET25b (+) vector at the BseRI restriction sites. The configuration of the αPD-1 (scFv) was NH2–VH–linker–VL–COOH, where VH and VL were the variable regions of the αPD-1 heavy chain and the αPD-1 light chain, respectively. Two mutations were introduced into αPD-1, VH, R54C and VL, G104C. The linker in αPD-1 was (GGGGS). The linker was also inserted between the ABD domain and the PE domain of ABD–PE. The coding genes of αPD-1–ABD–PE and αPD–1 PE were generated by fusing the synthesized genes using a previously described protocol. Meanwhile, a gene encoding the linker was inserted between the genes for αPD-1 and ABD–PE, and between the genes for αPD-1 and PE. The linker gene was generated by annealing the sense and antisense oligonucleotides of the gene (Eurofins Genomics). A hexahistidine tag (HisTag) was added at the N-terminus of αPD-1–ABD–PE and the control proteins to facilitate their purification; the GGGGS linker was inserted between the HisTag and the proteins. Accordingly, the coding gene of the HisTag was also fused to the coding genes of these proteins. The sizes of the final constructs were confirmed by agarose gel electrophoresis after cleavage from the pET25b (+) vector by double digestion with BamHI and Xbal. The sequences of the genes were verified by DNA sequencing (Geneviz).

Protein expression and purification. The pET25b (+) vectors harbouring coding sequences for αPD-1–ABD–PE, αPD-1–PE, and αPD–1 PE were transferred into competent Shuttle T7 E. coli (New England Biolabs). These transformed E. coli were cultured in LB broth at 32°C until the absorbance at 595 nm (A595) of the culture reached 0.6. At that point, the cultured cells were induced with 1 mM final concentration IPTG (Gold Biotechnology) for protein expression and incubated for a further 18 h.

The cultured E. coli were collected by centrifugation at 4,700 rpm for 30 min. The collected cells were lysed by sonication using a 4 min pulse. The cell samples were kept on ice during sonication, and 1 mM PMSF (Gold Biotechnology) was added to the cell lysate to inhibit protein degradation. After sonication, the supernatant of the cell lysate was collected by centrifuging the lysed samples at 20,000g for 1 h. Next, imidazole powder was added to the supernatant to reach a final imidazole concentration of 20 mM. At the same time, HisPur Ni-NTA resin (Thermo Fisher Scientific) was equilibrated with 10 mM imidazole in PBS. The equilibrated resin was then incubated with the supernatant for 1 h at 4°C on a rotator mixer. After the incubation, the mixture was loaded on a column, and the impurities and endotoxins were removed by washing the column with 60 mM imidazole that contained 1% Triton X-114. The wash was repeated until protein concentrations in the eluent were very low (A280 < 0.01). The use of 1% Triton X-114 made it unnecessary to add detergent removal washes, which were described previously. A further elution step, Triton X-114 was removed by washing the column with 50 column volumes of 60 mM imidazole. Finally, the desired proteins were eluted from the column with 300 mM imidazole in PBS (pH = 8). Imidazole was removed using PD-10 desalting columns (GE Healthcare Life Sciences). The purity and residual endotoxin level of the resulting proteins were analysed by SDS-PAGE and PYROGENT single-test vials (Lonza).

SDS-PAGE analysis of proteins. Five micrograms of αPD-1, ABD–PE, αPD–1 PE, and αPD–1–ABD–PE were first reduced with 2-mercaptoethanol and denatured by
heating at 95°C for 5 min. Then, these denatured samples were analysed by 4–15% gradient SDS–PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue and photographed using an Alpha Innotech Fluorchem FC2 gel imaging system. The image was processed by adjusting the brightness and contrast for the entire image using GNU Image Manipulation Program (GIMP).

**Determination of the binding affinity of αPD-1–ABD–PE to EL4 cells.** One-million EL4 cells were incubated with Alexa Fluor 647-labelled αPD-1–ABD–PE and anti-CD11c IgG at a series of different concentrations at 4°C for 30 min. Then, the EL4 cells were analysed by flow cytometry for PD-1+ EL4 cell fractions. The fraction of PD-1+ EL4 cells was plotted against protein concentration and Ki values were obtained by fitting the curve using the sigmoidal dose-response model in GraphPad v.5. The half-maximal response value (EC50) was used as the Ki value. GraphPad was used to calculate the 95% confidence intervals (95% CI).

**Generation of PD-1-/- primary T cells.** Mouse spleen cells were collected and cultured in 96-well plates at 2 x 10^5 cells per ml in complete RPMI-1640. Then, concanavalin A (Con A) (InvivoGen) was added to the culture at 6.25 μg/ml to stimulate PD-1 expression on primary T cells. After 72 h stimulation, cells were collected. PD-1-/- primary T cells were naive spleen cells cultured in the complete RPMI-1640 media without Con A for 72 h. These primary T cells were stained with PE-conjugated CD3 antibody for flow cytometry analysis.

**Generation of PD-1-/- primary B cells.** Mouse spleen cells were collected and cultured in 96-well plates at 2 x 10^5 cells per ml in complete RPMI-1640. Then, 10 μg/ml AffiniPure F(ab’2), fragment of goat anti-mouse IgM, μ-chain specific (Jackson Immunoresearch, cat code 115–006–028) was added to the culture to stimulate PD-1 expression on primary B cells. After 72 h stimulation, the cells were collected. PD-1-/- primary B cells were naive spleen cells cultured in the complete RPMI-1640 media without anti-IgM for 72 h. These primary B cells were stained with PE-conjugated B220 antibody for flow cytometry analysis.

**Evaluation of cell binding and endocytosis.** αPD-1–ABD–PE or ABDE-PE was first labelled with Alexa Fluor 647 NHS Ester (Thermo Fisher Scientific). The labelled αPD-1–ABD–PE or ABDE-PE (100 nM) was incubated with 0.5 x 10^6 cells at 4°C or 37°C in FACS buffer (PBS with 0.1% fetal bovine serum (FBS)) for 30 min. After the incubation, unbound proteins were removed by centrifugation (350 g for 5 min). Finally, the MFI of the incubation mixture was determined by flow cytometry with a BD FACSCanto Analyzer (BD Biosciences). Specifically, for primary B cells, the MFI values of B-cell populations were analysed. B-cell populations were identified by staining the cell mixture with PE-labelled B220 antibody. For primary T cells, the MFI values of T-cell populations were analysed. T-cell populations were identified by staining the cell mixture with PE-labelled CD3 antibody.

**Binding inhibition study with PD-L1-Fc.** A fusion protein containing PD-L1 and human Fc (PD-L1–Fc; Sino Biological, 50010-M03H) was used to compete for surface PD-1 binding with αPD-1–ABD–PE. For binding inhibition studies, 500 nmol of PD-L1–Fc–Fluor 647-labelled αPD-1–ABD–PE were incubated with 0.5 x 10^6 cells for 30 min at 4°C or 37°C. Then, unbound proteins were washed away by centrifugation (350 g for 5 min) in FACS buffer. The cells were analysed by flow cytometry with a BD FACSCanto Analyzer (BD Biosciences).

**Albumin-binding study.** Mouse splenocytes were collected and cultured in 96-well plates at 10^6 cells per ml in complete RPMI-1640. Then, free or NHS-fluorescein-labelled (Thermo Fisher Scientific) albumin (97% pure, Sigma) and HSA (96% pure, Sigma) in a 1:1 molar ratio in 20 μl medium at 37°C for 24 h. After the incubation, the samples were analysed as described above. The measurement was also performed for untreated cells (the live control) for cells that were treated with 1% Triton X-100 (the dead control). The albumin values were calculated for different concentrations of αPD-1–ABD–PE or ABDE-PE control mixture using the following equation: cell viability (%) = (A_total−A_all昴ew)/(A_total−A_d昴ew), where A_total is total of control- or αPD-1–ABD–PE-treated cells, A_all昴ew is A_total of the live control, and A_d昴ew is A_total of the dead control ( Triton X-100-treated cells). Viability data was fitted to a sigmoidal dose-response model to determine IC50 and 95% CI using GraphPad v.5 (n = 6).

**In vivo EL4 depletion.** Five-million EL4 (PD-1+) cells were injected intravenously into the tail of C57BL/6. The injected mice were randomly assigned into three groups. Two hours later, the three groups were treated with a single dose of control- or αPD-1–ABD–PE (5 mg per kg body weight), a mixture of αPD-1 (scFv, 2.5 mg per kg body weight) and ABDE-PE (2.5 mg per kg body weight), or PBS intraperitoneally. At 72 h after the treatments, these mice were euthanized, and circulating lymphocytes were collected from them. The cells were stained with Alexa Fluor 647-labelled PD-1 antibody and analysed by flow cytometry with a BD FACSCanto Analyzer to determine the fractions of PD-1+ cells among circulating lymphocytes.

**Pharmacokinetics.** Five nanomoles of NHS-fluorescein-labelled (Thermo Fisher Scientific) αPD-1–ABD–PE or PD-1–PE was injected intraperitoneally into C57BL/6 mice. At each pre-determined time point (0.25, 1, 2, 4, 12, 24, or 48 h after dosing), a cohort of three mice was killed, and blood was collected for quantification of protein concentration. Plasma concentrations of the proteins were determined according to previously described method. Plasma protein concentrations were determined by a competitive ELISA method with a minimum detection limit of 5 ng/ml, and the IC50 and 95% CI were calculated. The albumin values were calculated for different concentrations of αPD-1–ABD–PE or ABDE-PE using the following equation: cell viability (%) = (A_total−A_all昴ew)/(A_total−A_d昴ew), where A_total is total of control- or αPD-1–ABD–PE-treated cells, A_all昴ew is A_total of the live control, and A_d昴ew is A_total of the dead control ( Triton X-100-treated cells). Viability data was fitted to a sigmoidal dose-response model to determine IC50 and 95% CI using GraphPad v.5 (n = 6).

**Disease delay study with a spontaneous T1D model.** Twelve-week-old female NOD mice were randomly assigned into three groups. The three groups were treated intraperitoneally, weekly, with αPD-1–ABD–PE (5 mg per kg body weight), a mixture of αPD-1 (scFv, 2.5 mg per kg body weight) and ABDE-PE (2.5 mg per kg body weight), or PBS until the mice were found to be diabetic. Blood glucose levels of these mice were monitored twice weekly using a control and UltraMini Blood Glucose Meter (LifeScan). Onset of T1D was confirmed for a mouse when its blood glucose concentration was greater than 25 mg dl⁻¹ for two consecutive measurements. Diabetes-free survival was

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analysed by the Kaplan–Meier method followed by the log-rank (Mantel–Cox) test using GraphPad Prism v.5.

**Disease delay study with a cyclophosphamide-accelerated T1D model.** Ten-week-old female NOD mice were injected intraperitoneally with cyclophosphamide (Santa Cruz Biotechnology) at 200 mg per kg (body weight). Two days later, these mice were randomly assigned into three groups. The three groups were treated intraperitoneally with αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD–PE (2.5 mg per kg (body weight)), or PBS every other day for a total of five treatments. The diabetes-free survival data were generated and analysed as described above.

**Disease delay study with a αPD-1–accelerated T1D model.** Ten-week-old female NOD mice were randomly assigned into three groups. The three groups were treated intraperitoneally with αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD–PE (2.5 mg per kg (body weight)), or PBS every other day for a total of five treatments. Two days after the last dose, 0.5 mg anti-PD-1 IgG (clone DRM1–14) was administered intraperitoneally to each mouse. Two days later, the mice were given four additional doses of αPD-1 IgG at two-day intervals with a dose of 0.25 mg per mouse per injection. The diabetes-free survival data were collected and analysed as described above.

**Examination of immune cells in pancreases, blood and peripheral lymphatic organs of mice with EAE.** Eight-week-old female NOD mice were randomly assigned into three groups. The three groups were treated intraperitoneally with one dose of αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD–PE (2.5 mg per kg (body weight)) or PBS. Three days later, these mice were euthanized and perfused with 30 ml PBS. Pancreases were collected and a single cell preparation was generated from each of the pancreases by mincing the pancreas tissue with scissors and digesting them with 1 mg ml$^{-1}$ collagenase IV at 37 °C for 30 min as described previously. The pancreatic cell preparations were filtered through a nylon mesh and dead cells were first stained with 3 μM DAPI for 10 min and then washed three times by centrifugation (300g for 5 min) to remove DAPI. Cells were then stained with PE-conjugated B220 antibody, PE-conjugated CD8 antibody, PE-Cy7-conjugated CD3 antibody, FITC-conjugated CD4 antibody, PE-conjugated FOXP3 antibody (all antibodies were purchased from Biolegend), and Alexa Fluor 647-conjugated PD-1 antibody (made in house). Lastly, 400,000 live cells per pancreatic cell preparation were analysed by flow cytometry with a BD FACSCanto Analyzer. To analyse immune cells in blood and peripheral lymphatic organs of these mice, blood, spleens and lymph nodes (accessory axillary lymph nodes and subilac lymph nodes) were collected. Red blood cells were lysed with ammonium chloride–ACE (ACK) lysing buffer. The remaining cells were pelleted by centrifugation at 300g for 5 min, and stained with DAPI (3 μM), PE-conjugated B220 antibody, PE-Cy7-conjugated CD3 antibody, FITC-conjugated CD4 antibody, PE-conjugated FOXP3 antibody, and Alexa Fluor 647-conjugated PD-1 antibody. Lastly, 10,000 live cells per cell preparation were analysed by flow cytometry with a BD FACSCanto Analyzer.

**Disease reversal study in an EAE model.** Ten-week-old female C57BL/6 mice were subcutaneously immunized with 0.2 mg MOG35–55 peptide (MEVGWYRSPFSRVHLYRNGK, Biomatik) per mouse. The peptide was emulsified in 0.2 ml complete Freund's adjuvant (CFA, Sigma). Four hours later, the mice were injected intraperitoneally with 200 ng pertussis toxin (List Biological Labs), and an additional 200 ng pertussis toxin 24 h later. The immunized mice were examined for paralysis symptoms every other day from the ninth day after immunization. The symptoms were scored on the basis of a common standard: 0.0, limp tail or isolated weakness of gait without limp tail; 2.0, partial hind-leg paralysis; 3.0, total hind-leg or partial hind- and front-leg paralysis; 4.0, total hind-leg and partial front-leg paralysis; 5.0, moribund or dead animal. At the same time, the immunized mice were randomly assigned into three groups; each group would receive one of the three treatments: αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD–PE (2.5 mg per kg (body weight)), or PBS. When an immunized mouse developed severe EAE (clinical score 3.0), this mouse would be immediately treated according to its group assignment. All the treated mice were monitored continuously until they reached their humane endpoint or the end of the study.

**Examination of immune cells in pancreases, blood and peripheral lymphatic organs of mice with EAE.** Ten-week-old female C57BL/6 mice were induced with MOG35–55 peptide (incomplete Freund's adjuvant, Sigma). Seven days after the first immunization, the mice were immunized a second time. Ten days after the second immunization, the mice were killed, and an enzyme-linked immunosorbent assay (ELISA) was performed on the sera. The IgM levels were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described with some modification. Specifically, serum dilutions from 10$^{-1}$ to 10$^{-5}$ were applied to ELLA plates pre-coated with DNP–BPA (T thermo Fisher Scientific Inc) (1 μg per well, 4 °C, overnight). After unbound IgM was washed away, a secondary antibody, anti-IgM (μ chain)–horseradish peroxidase conjugate (Jackson ImmunoResearch) (1:5,000 diluted in PBS with 1% BSA) was added to the plates. After a 1 h incubation at room temperature, the remaining unbound secondary antibody was washed away, and a TMB substrate (Biocolor) was added to the plates. The plates were then incubated in the dark at room temperature for 20 min for the colourimetric reaction to develop. The reaction was stopped with 2 M H$_2$SO$_4$. Both A$_{450}$ and A$_{570}$ (background) were measured. The same diluted serum samples were also applied to ELLA plates pre-coated with the BSA control (Sigma) (1 μg per well, 4 °C, overnight) to determine the non-specific binding of IgM to the plates. All other procedures were the same as for the DNP-Ficoll sample. The same experiment was also performed with 10-week-old female C57BL/6 mice.

**Evaluation of CTL responses.** Ten-week-old female NOD mice were randomly assigned into four groups and treated with one dose of αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD–PE (2.5 mg per kg (body weight)), PBS, or cyclophosphamide (200 mg per kg (body weight)). Two days after the treatment, the mice were euthanized and perfused with 30 ml PBS. Mononuclear cells were harvested and analysed by flow cytometry. Dead cells were first stained with 3 μM DAPI for 10 min, and then washed 5 times by centrifugation (300g for 5 min) to remove DAPI. Cells were then stained with PE-conjugated B220 antibody, PE-conjugated CD8 antibody, PE-Cy7-conjugated CD3 antibody, FITC-conjugated CD4 antibody, PE-conjugated FOXP3 antibody (antibodies were purchased from Biolegend), 1-Ab MOG35–55–PE repressor (provided by NIH repressor care facility), and Alexa Fluor 647-conjugated PD-1 antibody. Lastly, 200,000 live cells per cell preparation were analysed by flow cytometry with a BD FACSCanto Analyzer. To analyse immune cells in blood and peripheral lymphatic organs of these mice, the blood, spleens and lymph nodes (accessory axillary lymph nodes and subilac lymph nodes) were collected. Red blood cells were lysed with ACK lysing buffer. The remaining cells in the samples were pelleted by centrifugation at 300g for 5 min, and then stained with one dose of each of the following antibodies: PE-conjugated CD8 antibody, PE-Cy7-conjugated CD3 antibody, FITC-conjugated CD4 antibody, PE-conjugated FOXP3 antibody, and Alexa Fluor 647-conjugated PD-1 antibody. Lastly, 10,000 live cells per cell preparation were analysed by flow cytometry with a BD FACSCanto Analyzer.

**Fraction analysis of peripheral lymphocytes in healthy NOD and C57BL/6 mice.** Ten-week-old female NOD mice were randomly assigned into four groups and treated with one dose of αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD–PE (2.5 mg per kg (body weight)), PBS, or cyclophosphamide (200 mg per kg (body weight)). Two days after the treatment, the mice were euthanized and perfused with 30 ml PBS. Mononuclear cells were harvested and analysed by flow cytometry. Dead cells were first stained with 3 μM DAPI for 10 min, and then washed 5 times by centrifugation (300g for 5 min) to remove DAPI. Cells were then stained with PE-conjugated B220 antibody, PE-conjugated CD8 antibody, PE-Cy7-conjugated CD3 antibody, PE-conjugated FOXP3 antibody, and Alexa Fluor 647-conjugated PD-1 antibody. Lastly, 10,000 live cells per cell preparation were analysed by flow cytometry with a BD FACSCanto Analyzer. The same experiment was conducted on 10-week-old female C57BL6 mice.

**Evaluation of humoral responses.** Ten-week-old female NOD mice were randomly assigned into four groups and treated once or five times with αPD-1–ABD–PE (5 mg per kg (body weight)), a mixture of αPD-1 (scFv, 2.5 mg per kg (body weight)) and ABD-PE (2.5 mg per kg (body weight)), PBS, or cyclophosphamide (200 mg per kg (body weight)). The same experiment was also performed with 10-week-old female C57BL/6 mice.
Data availability
The authors declare that all other data supporting the findings of this study are available within the paper and its Supplementary Information. Source data for the figures and encoding genes are available at figshare (https://figshare.com/s/0f10f3582c9e9165a).

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Author contributions
P.Z. and M.C. wrote the manuscript with significant suggestions from S.G.Z. and S.J.F. R.S.F., P.Z. and M.C. designed all experiments and analysed all experimental data. S.J.F. and X.H. contributed to the design of the T1D studies; R.S.F. contributed to the design of the EAE studies; Z.Z. generated PD-1− EL4 cells; Y.C. assisted with pharmacokinetics analysis. P.Z. designed and prepared immunotoxin with assistance from S.D. P.Z. characterized immunotoxin in vitro and in vivo. P.W. contributed to the design of ABD and the related studies. H.Y. provided the αPD-1 hybridoma and guidance on PD-1 biology. All authors discussed the results and commented on the manuscript.

Competing interests
M.C., P.Z. and P.W. have a pending patent application related to this work.

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Software and code

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- Data analysis: Phoenix WinNolin software, version 8.0 for PK analysis, Graphpad Prism V5 for other analyses.

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| Sample size | The sample sizes of this study were determined on the basis of similar published studies. For most in vivo studies, the experimental groups have 5 or 6 samples. These sizes ensure a parametric test with 90% power and a significance level of 0.05. |
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| Data exclusions | No data were excluded. |
| Replication | All data were generated from at least two repeated experiments, except for Figure 3c. |
| Randomization | All mice/experimental subjects were randomly assigned into experimental groups. |
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| n/a | Involved in the study |
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| ☒ | Eukaryotic cell lines |
| ✓ | Palaeontology |
| ☐ | Animals and other organisms |
| ☐ | Human research participants |
| ☐ | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

Capture anti-mouse IFN-γ mAb (clone: R4-6A2) and biotinylated, detection anti-mouse IFN-γ mAb (clone: XMG1.2-Biotin) were purchased from BioLegend, San Diego, CA. Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgM and Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) were purchased from Jackson ImmunoResearch Inc. Anti-mouse CD3 antibody (clone: 145-2C11) was purchased from BioCell, West Lebanon, NH. FITC-, PE-, APC- or biotin-conjugated monoclonal antibodies to Foxp3 (clone: MF-14), B220 (clone: RA3-6B2), CD4 (clone: GK1.5), and CD8 (clone: 53-6.7) were purchased from BioLegend, San Diego, CA.

**Validation**

All antibodies are validated by the commercial source.

### Eukaryotic cell lines

**Policy information about** [cell lines](#)

**Cell line source(s)**

Cell lines were purchased from ATCC.

**Authentication**

Cell lines were verified: EL4 cells (surface marker, morphology), B16-F10 (morphology, in vivo growth). We specifically verified the PD-1 expression of these cell lines.

**Mycoplasma contamination**

Cell lines were tested for mycoplasma contamination and found to be negative of mycoplasma contamination.

**Commonly misidentified lines (See ICLAC register)**

No commonly misidentified cell lines were used.

### Animals and other organisms

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**Laboratory animals**

NOD/ShiLt: Ten-to-eighteen-week-old female (from the Jackson Laboratory and from in-house breeding). Specific ages indicated in the manuscript.

CS7BL/6: Ten-week-old female (from Charles River).
| Field | Description |
|-------|-------------|
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight | Animal studies were conducted following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah. The group sizes of mice were determined and approved by the regulatory authorities for animal welfare after a balanced consideration of statistical and scientific needs and ethical aspects. All procedures related to animal studies are in compliance with relevant ethical regulations on animal research at the University of Utah. Note that full information on the approval of the study protocol must also be provided in the manuscript. |

**Flow Cytometry**

**Plots**

Confirm that:
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**Methodology**

| Sample preparation | Please refer to the Methods section for details. |
|-------------------|-----------------------------------------------|
| Instrument | BD FACS Canto Analyzer |
| Software | FlowJo v9.9 (Mac OS) |
| Cell population abundance | Cell populations were identified by their protein marker after the cells were stained with labelled antibodies. |
| Gating strategy | Cells were gated either against prepared negative samples (isotype control, no antibody staining) or the self-control negative populations. Supplementary Fig. 5 is one example. |

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