

CTLA-4 antibody-drug conjugate reveals autologous destruction of B-lymphocytes associated with regulatory T cell impairment

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Abstract

Germline CTLA-4 deficiency causes severe autoimmune diseases characterized by dysregulation of Foxp3⁺ Tregs, hyper-activation of effector memory T cells, and variable forms autoimmune cytopenia including gradual loss of B cells. Cancer patients with severe immune-related adverse events (irAE) after receiving anti-CTLA-4/PD-1 combination immunotherapy also have markedly reduced peripheral B cells. The immunological basis for B cell loss remains unexplained. Here we probe the decline of B cells in human CTLA-4 knock-in mice by using anti-human CTLA-4 antibody Ipilimumab conjugated to a drug payload emtansine (Anti-CTLA-4 ADC). The anti-CTLA-4 ADC-treated mice have T cell hyper-proliferation and their differentiation into effector cells which results in B cell depletion. B cell depletion is mediated by both CD4 and CD8 T cells and at least partially rescued by anti-TNF-alpha antibody. These data revealed an unexpected antagonism between T and B cells and the importance of regulatory T cells in preserving B cells.

eLife assessment

This study presents **valuable** information that an anti-CTLA-4 antibody drug conjugate transiently depletes circulating B cells in a mouse model. It shows how dysregulation of the T cell immune system can impact B cell homeostasis. The work will be of broad interest to immunologists and medical biologists, but the strength of evidence is **incomplete** at this time mainly because mechanistic insights into the B-cell reduction are insufficient.

Introduction

Foxp3 is a master regulator of regulatory T cells (Tregs) and its mutations result in fatal autoimmune disease in mice and human.^{1,2,3,4,5} Among many known functions, Foxp3 is a transcriptional factor for expression of CTLA-4, which is constitutively expressed in Tregs.^{6,7,8} *Ctla4* deletion in mice phenocopies that of Foxp3. In humans, CTLA-4 deficiency caused by autosomal heterozygous mutation of the CTLA-4 gene^{8,9,10} or mutations in recycling partner LPS-Responsive beige-like anchor (LRBA) protein¹⁰ are associated with severe autoimmune diseases. Although CTLA-4 can be expressed at lower levels in other cell types and has been suggested as a negative regulator for naïve T cell activation, lineage-specific deletion of the *Ctla4* gene in Foxp3⁺ Tregs results in development of systemic lymphoproliferation, fatal autoimmune disease, and potent tumor immunity.¹¹ These data are consistent with the notion that the predominant function of CTLA-4 is Treg-intrinsic.

A largely overlooked area is the cross-regulation between B cells and regulatory T cells. CTLA-4 conditional null mice and those with depletion of Tregs showed increase in germinal center B cells and heightened antibody responses with some reports showing a decrease B cell percentage.^{12,13,14,15} B cell loss is a common feature in genetic mutations of Foxp3, scurfy mice, Foxp3 knockout mice or Treg depletion.^{16,17,18,19,20,21} Among cancer patients who received immunotherapy, early change of circulating B cells of patients who received combination immunotherapy anti-CTLA-4/PD-1 correlated to irAE.²² The change in B cells included decline of circulating B cells, and increase in CD21 low B cells and plasmablasts.²²

The correlation between B cell loss and immune activation associated with defective Foxp3-CTLA-4 function remains unexplained. Since both CTLA-4 and Foxp3 are largely expressed outside of B cell compartment, and since this pathway is the master regulatory of Treg function, we hypothesized that B cell loss maybe associated with activation of T cells that are autodestructive of B cells. This hypothesis is noteworthy as no autodestructive T cells for B cells have been described. To test this hypothesis, we generated anti-CTLA-4 ADC and showed that the ADC caused selective depletion of Tregs in mice and a marked reduction of B cells. Remarkably, activation of CD4 and CD8 T cells is the underlying cause of B cell depletion. Our data explains the B cell loss associated with Foxp3 and CTLA-4 dysfunction and suggested an unexpected antagonism between T cells and B cells.

Results

CTLA-4 antibody-drug conjugate depleted regulatory T cells and B cells

Anti-CTLA-4 antibody Ipilimumab (Ipi) or human IgGfC (hIgGfC) control were conjugated to a well-known drug payload DM1, emtansine, to furnish the corresponding ADCs: Ipilimumab-DM1 (Ipi-DM1) and hIgGfC-DM1 with corresponding size shifts in the SDS-gel (**Figure 1A**). The drug to antibody ratio (DAR) was calculated based on experimentally determined extinction coefficient A280 & A252 for each antibody and reported values for DM1 (**Table S1**). The DAR from various conjugation is ~3.2 for Ipi-DM1 and ~1.7 for hIgGfC-DM1 control (**Table S1**), as expected based on the sizes of the proteins. The binding for Ipi-DM1 ADC was evaluated by ELISA binding to immobilized His-hCTLA-4 and by flow cytometry using hCTLA-4 expressing CHO cells (CHO-hCTLA-4). Ipi-DM1 binding was found comparable to the parent antibody Ipi (**Figures 1B**, **1C**). Specific killing of CTLA-4-expressing cells by Ipi-DM1 was assessed in human CTLA-4 expressing CHO (CHO-hCTLA-4) and wild type CHO (CHO-WT) cells in vitro (**Figure 1-figure supplement 1**),

which showed Ipi-DM1 reduced viability of CTLA-4 expressing CHO but not wild type cells. As expected, there was no change in viability for either CHO cell lines when treated with Ipi (**Figure 1-figure supplement 1** [↗](#)).

In order to evaluate the in vivo effects of CTLA-4 ADC on Tregs, we treated human CTLA-4 knock-in (*Ctla4^{h/h}*) mice with control hIgGFc or Ipi-DM1 ADC (**Figure 1D** [↗](#)). Peripheral blood was stained for flow cytometry and gated on CD45 and T-cell subset, including CD8, CD4, CD4 Foxp3⁺ and CD4 Foxp3⁻ subsets (**Figure 1-figure supplement 2A** [↗](#)). We found that Ipi-DM1 ADC significantly depleted Foxp3⁺ Tregs as indicated by the reduction in percentage and cell number of Foxp3⁺ CD4 T cells when compared to hIgGFc control (**Figure 1E** [↗](#)). In addition, total CTLA-4 levels and Ki67 staining in Tregs were decreased compared to control group (**Figures 1F** [↗](#), **1G** [↗](#)). In contrast, Ipi-DM1 ADC did not alter either % of CD4 Foxp3⁻ cells among CD45⁺ leukocytes or CTLA-4 levels on the subset, but did slightly decrease the cell number of CD4 Foxp3⁻ cells when compared hIgGFc control (**Figure 1-figure supplement 3A** [↗](#), **3B** [↗](#)). Unexpectedly, Ipi-DM1 ADC caused a dramatic decline of B cells as percentages of CD45 and cell number in the periphery (**Figure 1H** [↗](#)). B cells from mice treated with Ipi-DM1 exhibited higher proliferation than those from hIgGFc treated control mice (**Figure 1I** [↗](#)).

Since a dramatic loss of B cells and a slight decrease in CD4-nonTregs in Ipi-DM1 treated mice were observed, we sought ensure that this phenomenon was not a result of downstream release of payload drug DM1. As shown in **Figure 1-figure supplement 4A** [↗](#), hIgGFc-DM1 ADC (payload control) did not alter Treg percentage and cell number. A slightly higher level of CTLA-4 was noted in CD4 subset, while Treg proliferation did not change significantly compared to control (**Figure 1-figure supplement 4B** [↗](#), **4C** [↗](#)). No other effects on T cell subsets were noted (**Figure 1-figure supplement 4D** [↗](#), **4E** [↗](#)). Importantly, B cell number and proliferation were unaffected by hIgGFc-DM1 ADC treatment (**Figure 1-figure supplement 4F** [↗](#), **4G** [↗](#)). Additionally, to ensure B cell depletion was not directly caused by Ipi-DM1 ADC, we stained B220⁺ B cells and Foxp3⁺ Tregs for human CTLA-4 in both human CTLA-4 knock-in (*Ctla4^{h/h}*) and WT mice to evaluate expression of CTLA-4 in B cells. As expected, CTLA-4 was detected in Foxp3⁺ Tregs of *Ctla4^{h/h}* but not B cells (**Figure 1-figure supplement 5** [↗](#)).

We then investigated the impact of anti-CTLA-4 ADC in lymphoid organs. Treg percentage and absolute numbers were not impacted by Ipi-DM1 treatment compared to control in the spleen at day 9. The percent of B cells decreased significantly, although the reduction in absolute number of B cells was not statistically significant (**Figure 1-figure supplement 6A** [↗](#), **6B** [↗](#)). Flow analysis of lymph nodes after Ipi-DM1 treatment resulted in a significant increase in absolute cell numbers of Tregs and B cells and B cell percentage. (**Figure 1-figure supplement 6C** [↗](#), **6D** [↗](#)).

We then asked how Ipi-DM1 ADC induced Treg impairment impacted B cell lymphopoiesis. Bone marrow cells were stained for flow cytometry and gated on CD45 and T-cell subsets were defined as CD8 (CD8⁺B220⁻), CD4 (CD4⁺B220⁻), Tregs (CD4⁺B220⁻ Foxp3⁺) and CD4-nonTregs (CD4⁺B220⁻ Foxp3⁻) (**Figure 1-figure supplement 2B** [↗](#)). Ipi-DM1 depletion of Tregs was confirmed in the bone marrow (**Figure 1J** [↗](#)). Additionally, total CTLA-4 level in bone marrow Tregs decreased, however Ki67 staining did not change significantly compared to control group (**Figures 1K** [↗](#), **1L**). In contrast, Ipi-DM1 ADC did not alter bone marrow CD4-nonTreg cell percentage among CD45⁺, absolute cell number, or CTLA-4 level compared hIgGFc control (**Figure 1-figure supplement 3C** [↗](#), **3D** [↗](#)). FACS analysis of bone marrow B cells revealed the loss of mature B cells but not immature transitional type1 or Pre-pro/Pro/Pre B cells from Ipi-DM1 treated mice (**Figure 1M** [↗](#)-**1Q** [↗](#), **Figure 1-figure supplement 7** [↗](#)). Corresponding to mature B cell loss, the Pre-Pro/Pro/Pre B cell subtype percentage in B220 are enriched (**Figure 1P** [↗](#), **1Q** [↗](#), **Figure 1-figure supplement 7** [↗](#)). Additionally, CD21/35 expression level in the remaining mature B cells in Ipi-DM1 treated mice was lower than those from hIgGFc treated control mice (**Figure 1R** [↗](#)).

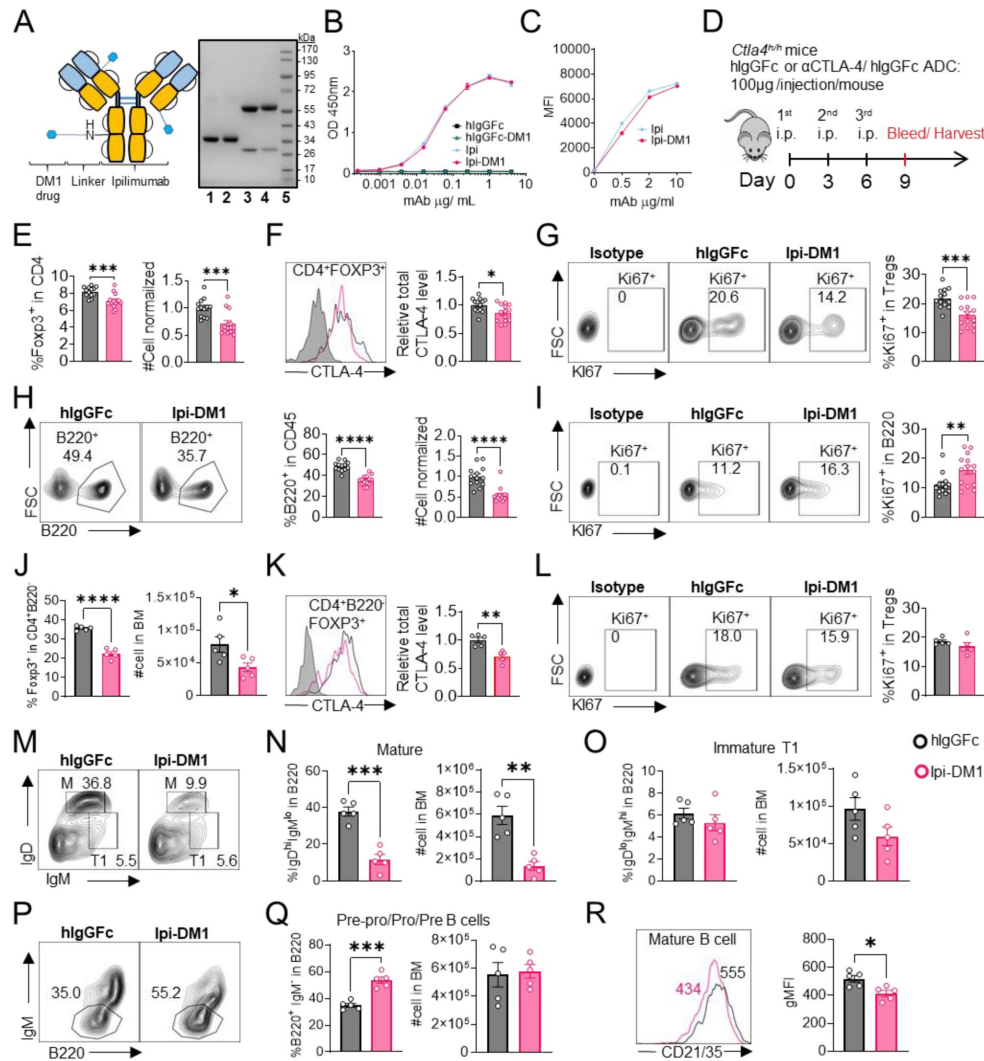


Figure 1.

CTLA-4 antibody-drug conjugate impairs regulatory T-cell and leads to B-cell depletion.

(A) SDS-Reducing gel showing size shift after DM1 conjugation. Lanes (left to right) are 1 hIgGFc, 2 hIgGFc-DM1 antibody-drug conjugate (ADC), 3 Ipilimumab (Ipi), 4 Ipilimumab-DM1 (Ipi-DM1) antibody-drug conjugate (ADC), and 5 Ladder. (B) ELISA binding of hIgGFc, hIgGFc-DM1, Ipi, and Ipi-DM1 to pre-coated 1 $\mu\text{g/mL}$ His-hCTLA-4 and detected with anti-hIgG-HRP (n=2). (C) Flow cytometry binding of Ipi and Ipi-DM1 to hCTLA-4 expressing CHO cells (CHO-hCTLA-4) and detected with anti-IgG-AF488. (D) Diagram of experimental design, *Ctla4^{h/h}* mice were treated intraperitoneally (i.p.) (100 $\mu\text{g}/\text{mouse}$) with hIgGFc or Ipi-DM1 every three days and mice were bled or harvested for bone marrow extraction on day 9 for downstream flow analysis. (E-I) flow data analysis of day 9 peripheral blood, Tregs defined ($\text{CD4}^+ \text{CD4}^+ \text{Foxp3}^+$) and B cells defined ($\text{CD45}^+ \text{B220}^+$). (E) % Foxp3 in CD4 and normalized cell number. (F) Relative CTLA-4 Level in Tregs. (G) % Ki67⁺ in Tregs. (H) FACS profiles depicting gating strategy after gating on CD45^+ and data summaries of % B220⁺ in CD45^+ and normalized cell number. (I) % Ki67⁺ in B cells. (J-R) Flow data analysis of day 9 bone marrow, Tregs defined ($\text{CD4}^+ \text{B220}^+ \text{Foxp3}^+$) and B220⁺ B cell subtypes defined mature (M) ($\text{IgD}^{\text{hi}} \text{IgM}^{\text{low}}$), immature transitional type 1 (T1) ($\text{IgD}^{\text{low}} \text{IgM}^+$), and Pre-pro/Pro/Pre B (B220⁺ IgM). (J) % Foxp3⁺ in $\text{CD4}^+ \text{B220}^-$ and absolute cell number. (K) Relative CTLA-4 Level in Tregs. (L) % Ki67⁺ in Tregs. (M) FACS profile gating of M and T1 B cells, (N) % of mature B cells in B220 and absolute cell number summaries, (O) % of T1 immature B cells in B220 and absolute cell number summaries. (P) FACS profile gating of Pre-pro/Pro/Pre B cells, (Q) % of Pre-pro/Pro/Pre B cells in B220 and absolute cell number summaries. (R) CD21/35 expression in mature B cells in bone marrow. (B, C) Representative data of two or more repeats. (E-I) Data combined from two independent experiments (n=13-14). (J-R) Data representative of two independent experiments (n=5). Data analyzed using an unpaired two-tailed Student's t test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

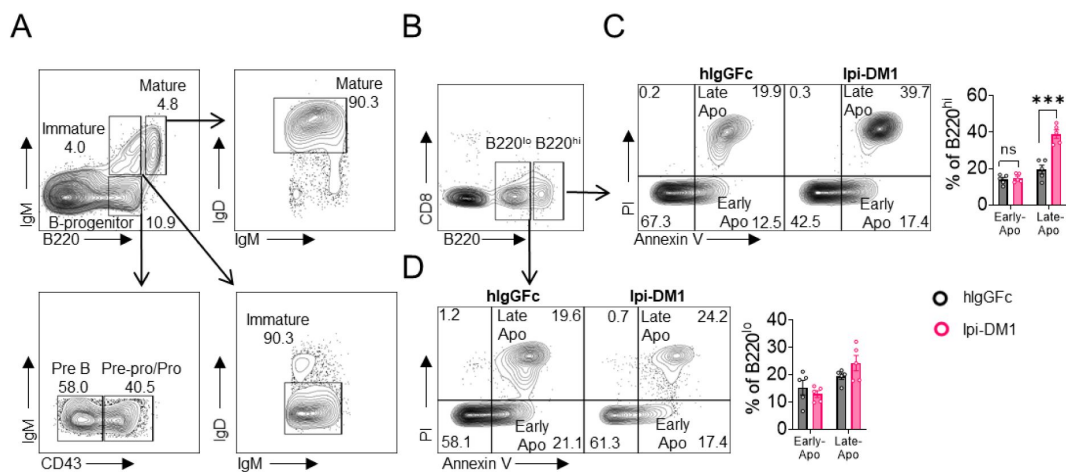


Figure 2.

CTLA-4 antibody-drug conjugate increases apoptosis in mature B cells in bone marrow.

Flow analysis of B cell apoptosis on day 9 after treatment with hlgGFc or Ipi-DM1 ADC. (A) FACS profile defining B220^{hi} and B220^{lo} B cell populations in bone marrow (B) Gating strategy for B220^{hi} (mature B cells) and B220^{lo} (progenitor/immature B cells) for apoptosis analysis. (C, D) FACS profiles and summaries depicting apoptosis of B cells in (C) B220^{hi} bone marrow cells, (D) B220^{lo} bone marrow cells. Data representative of two independent experiments (n=5) and analyzed using an unpaired two-tailed Student's t test and represented as mean $\pm$ SEM. Non-significant (ns) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

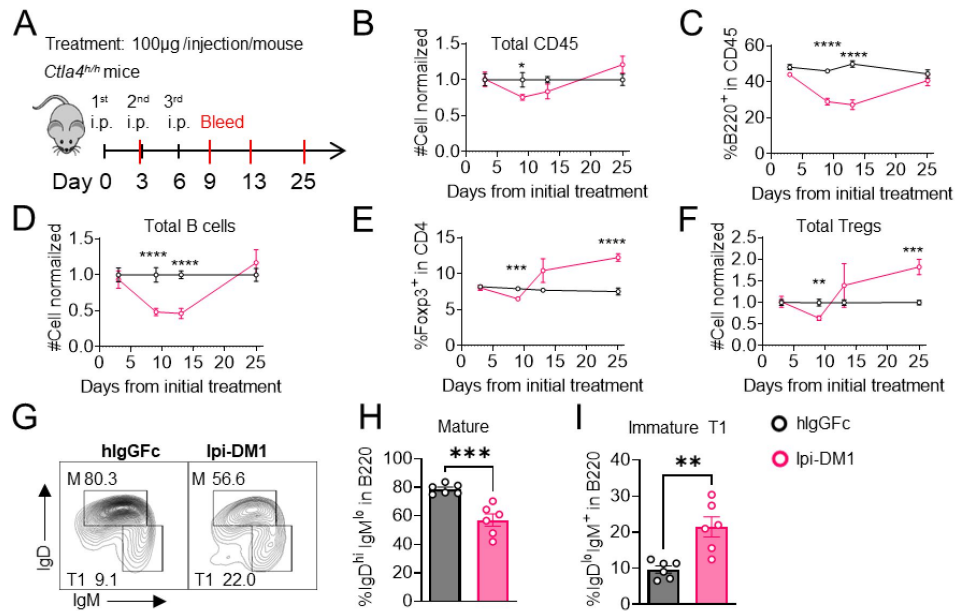


Figure 3.

B-cell depletion is transient and correlated with a decrease in regulatory T-cell.

(A) Diagram of experimental design, *Ctla4^{h/h}* mice were treated intraperitoneal (i.p.) (100 µg/mouse) with hlgGFc or Ipi-DM1 every three days and mice were bled on day 3,9,13, 25 for downstream flow analysis. (B-F) Flow data of time course of CD45, B, and Treg cells in peripheral blood, (B) CD45 normalized cell number, (C) % of B-cells in CD45, (D) B-cell normalized cell number, (E) % Foxp3⁺ Tregs in CD4, (F) Treg normalized cell number. (G-J) Flow analysis of B-cell subtypes from day 13 peripheral blood. (G) FACS profile of Mature (M) (IgD^{hi} IgM^{low}), and immature Transitional type 1 (T1) (IgD^{low} IgM⁺) B cell subtypes after gating on CD45⁺ B220⁺ B cells. (H) % of mature B cells in B220. (I) % of T1 B cells in B220. (B-F) Data combined from two independent experiments (n=10). (G-I) Data representative of two independent experiments (n=6). Data were analyzed by an unpaired two-tailed Student's t test and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

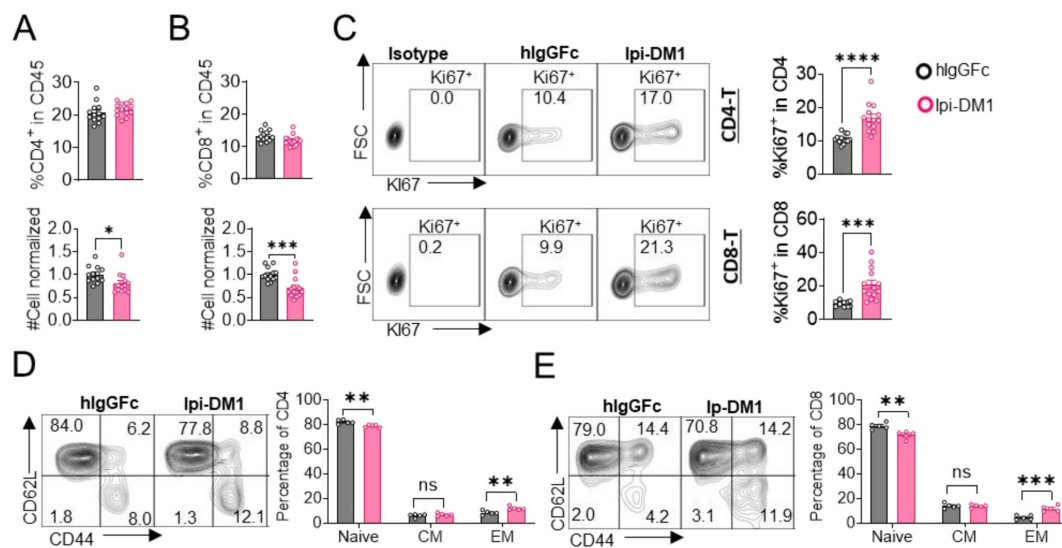


Figure 4.

CTLA-4 antibody-drug conjugate leads to T-cell activation.

Flow data analysis of peripheral blood from *Ctla4^{h/h}* mice on day 9 after treatment with hIgGFc or Ipi-DM1 ADC. (A) % CD4⁺ in CD45 and normalized cell number. (B) % CD8⁺ in CD45 and normalized cell number. (C) % Ki67⁺ in CD4 and CD8 T cells. (D, E) FACS profiles and summaries depicting the increase in effector T-cells after Ipi-DM1 treatment, naïve (Q1: CD44^{low} CD62L^{hi}), central memory (Q2: CD44^{hi} CD62L^{hi}) and effector (Q3: CD44^{hi} CD62L^{low}), phenotype of CD4⁺ T cells (D), and CD8 T cells (E). (B-C) Data combined from two independent experiments (n=13-14). (D-E) Data representative of two independent experiments (n=5). Data analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. Non-significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

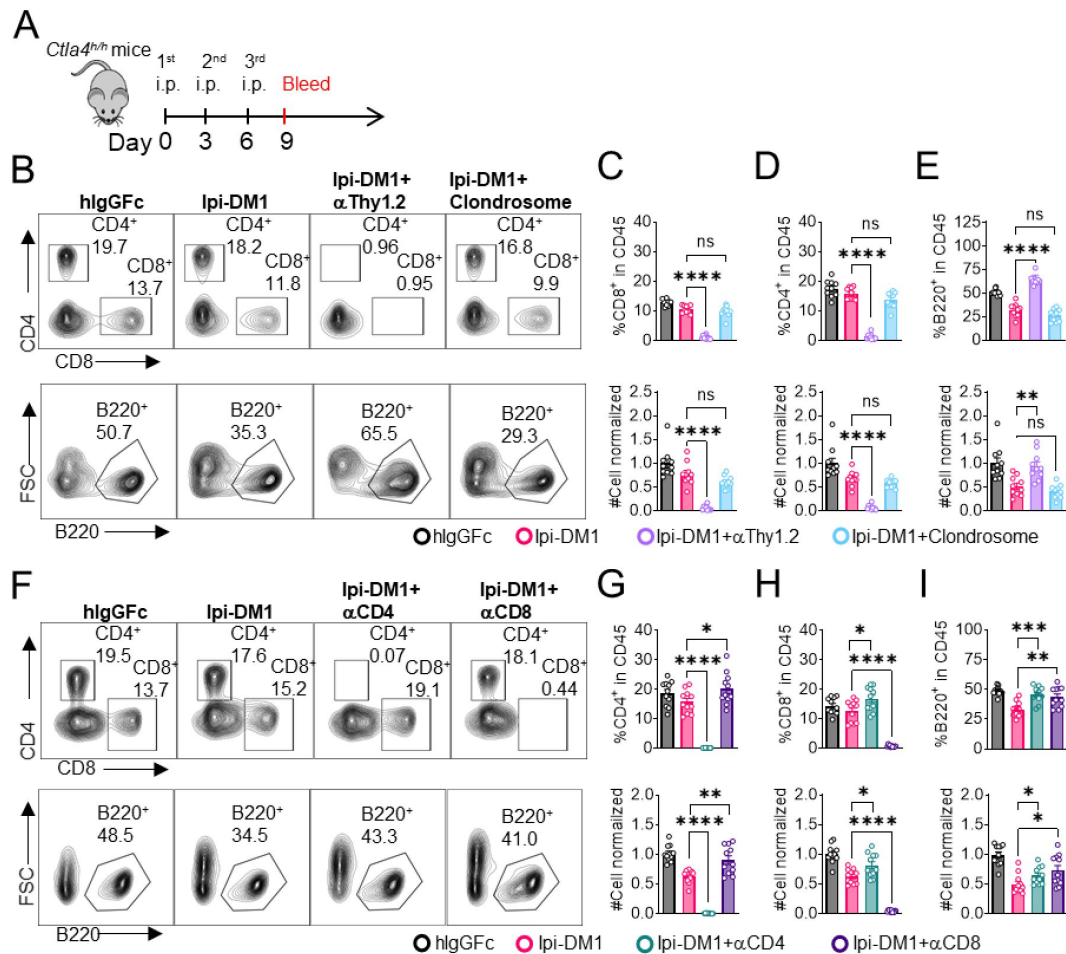


Figure 5.

B-cell depletion depends on T-cells but not macrophage.

(A) Diagram of experimental design, male or female *Ctlα4^{h/h}* mice were treated intraperitoneal (i.p.) (100 μg/mouse) with hlgGFc or Ipi-DM1 with/out (T-cell depleting antibody 100 μg/mouse of anti-Thy1.2 or 150 μL/mouse of clondrosome or depleting antibody CD4 or CD8 100 μg/mouse) every three days and mice were bled on day 9 for downstream flow analysis. (B) FACS profiles depicting gating strategy after gating on CD45 for CD8 & CD4 T (top panel) and B cells (bottom panel). (C-E) Data summaries from FACS profile (panel B), (C) % CD4⁺ in CD45 and normalized cell number, (D) % CD8 in CD45 and normalized cell number, and (E) % B220⁺ in CD45 and normalized cell number. (F) FACS profiles depicting gating strategy after gating on CD45 for CD8 & CD4 T (top panel) and B cells (bottom panel). (G-I) Data summaries from FACS profile (panel F), (G) % CD4⁺ in CD45 and normalized cell number, (H) % CD8⁺ in CD45 and normalized cell number, and (I) % B220⁺ in CD45 and normalized cell number. (B-E) Data combined from two independent experiments (n=10) and analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons test and represented as mean ± SEM. Non-significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (F-I) Data combined from two independent experiments (n=11) and analyzed by unpaired two-tailed Student's t test and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

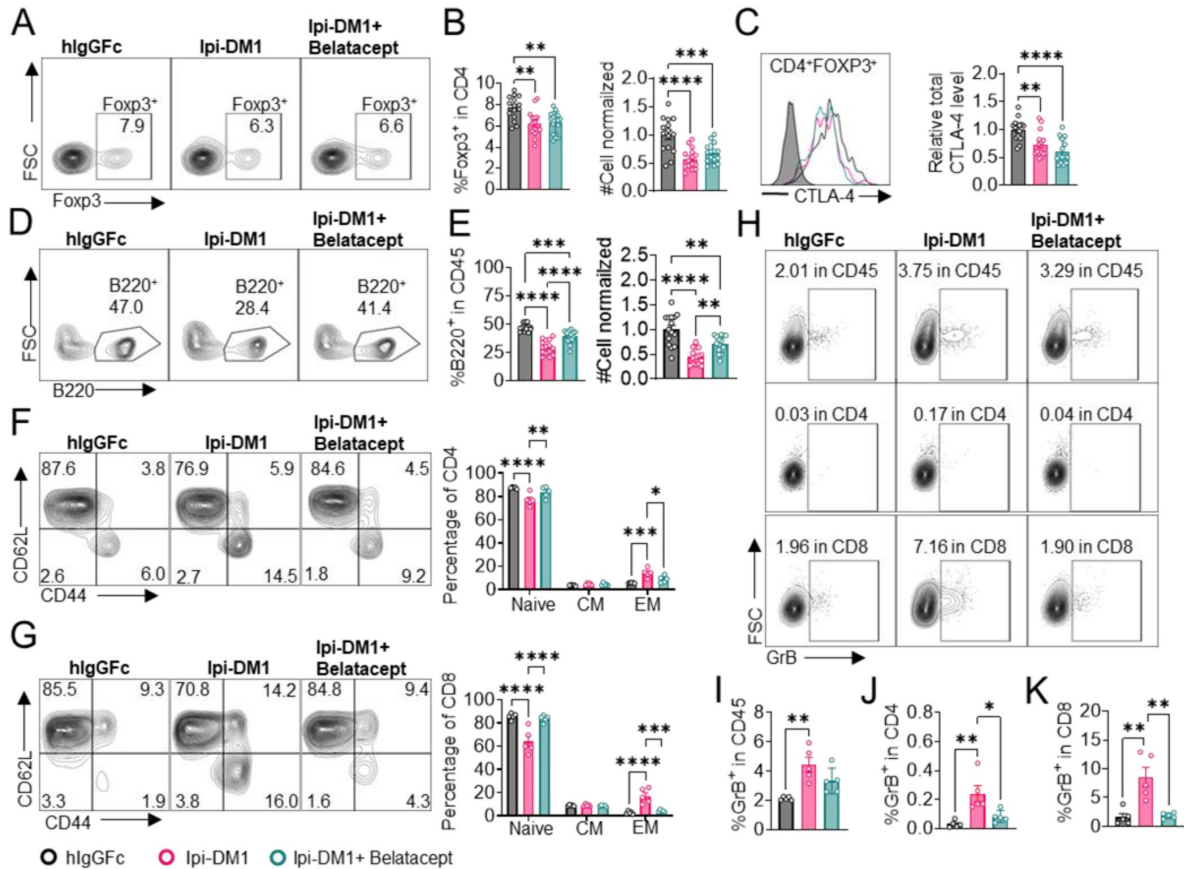


Figure 6.

Mutant soluble CTLA-4-Ig rescues B-cell.

Ctla4^{h/h} mice were treated intraperitoneal (i.p.) (100 µg/mouse) with hlgGFc or Ipi-DM1 with/out (100 µg/mouse of Belatacept) every three days for total of three doses and mice were bled on day 9 for downstream flow analysis. (A, D) FACS profiles depicting gating strategy after gating on CD45 for (A) Tregs (% Foxp3⁺ in CD4 T cells) and (D) B cells (% B220⁺ in CD45). (B) % Foxp3⁺ (Tregs) in CD4 and normalized cell number. (C) Representative FACS profile of CTLA-4 expression in Tregs (CD4⁺ Foxp3⁺) and Relative CTLA-4 summary. (E) % B220⁺ in CD45 and normalized cell number. (F-G) FACS gate and summaries showing mutant CTLA-4-Ig can decrease effector memory T-cells associated with Ipi-DM1 treatment, naïve (Q1: CD44^{low} CD62L^{hi}), central memory (Q2: CD44^{hi} CD62L^{hi}) and effector (Q3: CD44^{hi} CD62L^{low}), + + phenotype of CD4⁺ T cells (F), and CD8 T cells (G). (H) FACS of GranzymeB gating in CD45 (Top panel), CD4 (middle panel), and CD8 (bottom panel). (I-K) GranzymeB expression summaries in (I) CD45, (J) CD4, and (K) CD8 cells. (A-E) Data combined from three independent experiments (n=15). (F-K) Data representative of two independent experiments (n=5). Data analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons test and represented as mean ± SEM. Non-significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

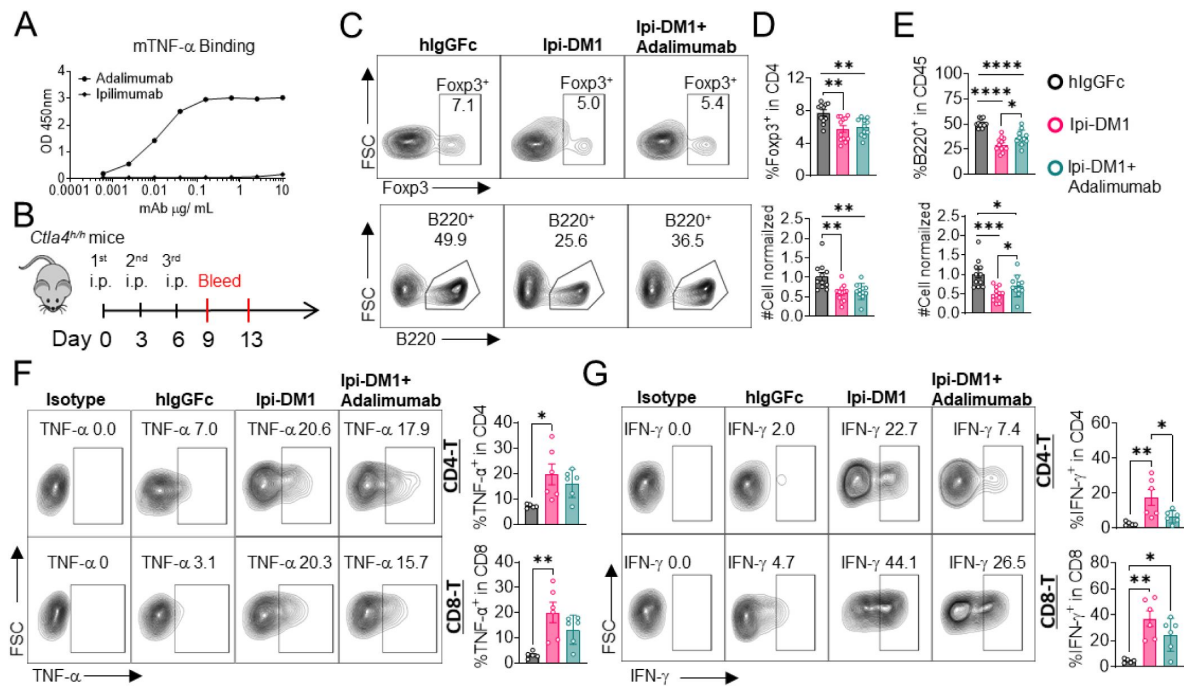


Figure 7.

B-cell depletion is partially rescued by anti-TNF-alpha.

(A) ELISA binding of clinical grade drug Adalimumab (Humira) to pre-coated 1 μ g/mL mouse-TNF-alpha and detected with anti-hIgG-HRP, Ipilimumab negative control. (B) Diagram of experimental design, male *Ctla4^{h/h}* mice were treated intraperitoneal (i.p.) (100 μ g/mouse) with hlgGfC or Ipi-DM1 with/out (100 μ g/mouse of Adalimumab) every three days for total of three doses and mice were bled on day 9 and 13. (C) FACS profiles depicting gating strategy after ⁺ gating on CD45 for Tregs (% Fop3⁺ in CD4) (top panel) and B cells (% B220⁺ in CD45) (bottom panel) from day 9 peripheral blood. (D) % Fop3⁺ (Tregs) in CD4 and normalized cell number. (E) % B220⁺ in CD45 and normalized cell number. (F, G) Day 13 peripheral blood post red blood lysis by ACK buffer were cultured and stimulated in the presence of Iononmycin/PMA, and GolgiPlug for 4 hrs followed by intracellular cytokine detection (TNF-alpha, IFN-gamma) in CD4 and CD8 T cells, (F) TNF-alpha, and (G) IFN-gamma. (C-E) Data combined from two independent experiments (n=11-12) and analyzed using an unpaired two-tailed Student's t test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (F, G) Data representative of two independent experiments (n=5-6) and analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

We then investigated B cell apoptosis in peripheral blood and lymphoid organs (**Figure 2**, **Figure 2-figure supplement 1**). Only mature bone marrow B220^{hi} B cells showed increase in apoptosis from Ipi-DM1 treatment, while progenitor/immature bone marrow B220^{lo} B cells were similar to control group. Blood and spleen B cell apoptosis were similar to hIgGFC control group, while lymph node B cell apoptosis was lower than the control group. Thus, the loss of B cells in the peripheral blood is likely due to loss of mature B cells in the bone marrow, and that such loss correlates with Treg depletion.

Taken together, data in **Figure 1** & **2** showed that Ipi-DM1 can impair Treg function by depleting Tregs, preferentially the proliferating Tregs with higher CTLA-4 levels, and loss of B cells in the blood and bone marrow while B cell progenitors are not impacted. B cell death increase is observed in only B220^{hi} bone marrow B cells, which are predominantly mature B cells.

B-cell depletion correlates with Treg impairment by Ipi-DM1 and increases immunoglobulins

To investigate the kinetic relationship between the decline of B cells and Treg impairment, human CTLA-4 knock-in (Ctla4^{h/h}) mice were treated with control hIgGFC or Ipi-DM1 and bled according to schedule diagram in **Figure 3A**. We observed that peripheral blood samples from mice treated with Ipi-DM1 had a gradual decrease in total leukocytes compared hIgGFC control group followed by a full recovery by Day 25 (**Figure 3B**). The large decrease of CD45 cells in Ipi-DM1 group is predominantly attributed to the decline of B cells, which plateaus on days 9 and 13 and recovers by day 25 (**Figures 3C**, **3D**) in correlation with the kinetics of Tregs (**Figure 3E**, **3F**). Similar to the bone marrow data in **Figure 1**, peripheral blood mature B cells decreased and transitional type1 B cells were enriched in Ipi-DM1 group compared hIgGFC control (**Figure 3G**–**3I**). Additionally, Treg impairment by Ipi-DM1 increases immunoglobulins especially IgE (**Figure 3-figure supplement 1**). The lack of Treg depletion in the spleen and lymph nodes at these time points remains to be explained, although significant immune activation, as suggested by splenomegaly and adenopathy (**Figure 3-figure supplement 2**), may result in production of cytokines that drive Treg proliferation.

Depletion of T-cells but not macrophage rescues B-cells from ablation by Ipi-DM1

We then evaluated how Ipi-DM1-mediated impairment of Treg function affects total CD4 and CD8 T cells in peripheral blood. Mice treated with Ipi-DM1 ADC did not affect the total percentage of CD4 or CD8 T cells among CD45⁺ leukocytes, but the cell number of both cell types decreased (**Figure 4A**, **4B**). Analysis of lymphoid organ T cells showed differing T cell absolute number phenotype where bone marrow, thymus and spleen T cells were similar to control hIgGFC while lymph node T cells increased (**Figure 4-figure supplement 1**). Staining of Ki67 in CD4 and CD8 revealed a greater percentage of T cells in hyper-proliferative state in blood and lymphoid organs (**Figure 4C**, **Figure 4-figure supplement 2**). Alternatively, mice treated with hIgGFC-DM1 payload control did not change CD4 or CD8 percentage of CD45, cell numbers and Ki67 staining compared to hIgGFC control in the periphery (**Figure 4-figure supplement 3**).

We then analyzed the functional subsets of T cells in mice that received control hIgGFC or Ipi-DM1 at day 9. Using CD44 and CD62L markers, we observed an expansion of effector memory T cells (CD44^{hi}CD62L^{low}) in Ipi-DM1 group for both CD4 and CD8 T cells (**Figures 4D**, **4E**). Correspondingly, the frequency of naive T cells was reduced for Ipi-DM1 while central memory T cells were unchanged (**Figures 4D**, **4E**). These results show that CTLA-4 ADC can impair Treg function thereby resulting an increase in effector memory T cells and a hyper-proliferative state similar to animals that lack the expression of CTLA-4 or Foxp3.

Since B cells are devoid of CTLA-4, Ipi-DM1 must have induced other effector cells that are directly responsible for B cell depletion. To understand which cell types are responsible for the destruction of B cells, we depleted either T cells or macrophages using either anti-Thy1.2 mAb or clondrosome. *Ctla4^{h/h}* mice were treated with control hIgGfC, Ipi-DM1, Ipi-DM1 in combination with anti-Thy1.2, or Ipi-DM1 in combination with clondrosome and bled on day 9 (**Figure 5A** [↗](#)). As shown in the **Figure 5B** [↗](#), top panel, anti-Thy1.2 mAb efficiently depleted total T cells while macrophage depletion by clondrosome had no effect (**Figure 5B** [↗](#) top panel, 5C, 5D). Additionally, combination of Ipi-DM1 with T cell depleting antibody resulted in a remarkable rescue of B cells as indicated by percentage B220 in CD45 and cell number while combination with macrophage depletion did not rescue the B cells (**Figures 5B** [↗](#) bottom panel, 5E).

In order to understand which subsets of T cells played a critical role in the T cell mediated destruction of B cells, we treated *Ctla4^{h/h}* mice with hIgGfC control, Ipi-DM1, Ipi-DM1 with CD4 depleting antibody, or Ipi-DM1 with CD8 depleting antibody (**Figure 5A** [↗](#)). Both CD4 and CD8 depleting antibodies provide efficient depletion (**Figure 5F** [↗](#), top panel) of their respective targeted T cell. Depletion of either CD4 or CD8 T cells results in B cell increase (**Figures 5F** [↗](#) bottom panel, 5I). Interestingly, the cell number of CD4 and CD8 T cells increase reciprocally by depletion of the other subsets (**Figures 5G** [↗](#), **5H** [↗](#)).

Taken together our CTLA-4 antibody-drug conjugate Ipi-DM1 can impair Treg function, which results in a T cell mediated destruction of B cells. Whole T cell depletion with anti-Thy1.2, depletion of CD4 or CD8 T cells rescued B cells from abrogation by Ipi-DM1.

B-cell rescue by Belatacept suggest a role for B7-CD28 interaction in B-cell depletion

The process for T cell activation requires two signals. The first signal is the binding of T-cell receptor (TCR) to antigen-bound major histocompatibility complex (MHC) and the second signal is a costimulatory molecule CD28 binding with B7-1/2 (CD80/86) on the antigen-presenting cell. Having shown that the destruction of B cell can be prevented by total depletion of T cell, we sought to rescue the B cells by breaking the CD28-B7 signal with a soluble CTLA-4 that can bind to B7 but not Ipilimumab or Ipi-DM1 ADC. As shown in **Figure 6-figure supplement 1** [↗](#), Abatacept can neutralize Ipilimumab/ADC while Belatacept does not.

Furthermore, we previously showed that human CTLA4-Ig or mutants can bind to murine B7-1/2 (CD80/86) and block their binding to murine CD28 efficiently. ^{23,24} [↗](#) Therefore, Belatacept can be used to test if activation of T cells is required for B cell depletion.

We treated mice with Ipi-DM1 to impair Treg function and used Belatacept to break CD28/B7 signal to block T cell activation. Briefly, *Ctla4^{h/h}* mice were treated with hIgGfC or Ipi-DM1 with/out Belatacept and bled on day 9 for flow analysis. Foxp3⁺ Tregs percentage in CD4 and cell number decreased similarly for Ipi-DM1 with/out Belatacept compared hIgGfC control (**Figures 6A** [↗](#), **6B** [↗](#)). Additionally, Belatacept did not impact Ipi-DM1 to target CTLA-4 expressing Tregs, as Ipi-DM1 with/out Belatacept had similar total CTLA-4 level reduction compared to control group (**Figure 6C** [↗](#)). However, Belatacept rescued B cells from Ipi-DM1-mediated depletion (**Figures 6D** [↗](#), **6E** [↗](#)). Correspondingly, Belatacept reduced effector memory T cells (**Figures 6F** [↗](#), **6G** [↗](#)) as well as granzyme B and IFN- γ expression in CD4 and CD8 T cells compared to Ipi-DM1 treated group (**Figures 6H** [↗](#)-**6K** [↗](#), **Figure 6-figure supplement 2** [↗](#)).

Collectively, the data presented here show that Ipi-DM1 can impair Treg function resulting in T cell activation as indicated by increase in effector memory T cell markers, GranzymeB, and cytokine production thereby resulting in the loss of B cells. However, under Ipi-DM1 induced Treg impairment the addition of mutant soluble CTLA-4-Ig, Belatacept, can reduce T cell activation and thereby preserving B cells.

B-cell depletion is partially rescued by anti-TNF-alpha

Since T cells are the effector cells responsible for B cell abrogation, it is of great interest to evaluate the molecular mechanism by which B cells are eliminated by T cells. To address this issue, we tested if either FasL or TNF-alpha, which are produced by activated T cells are responsible. To answer this question, *Ctla4^{h/h}* mice were treated with hIgGFc control or Ipi-DM1 with/out Adalimumab a human anti-TNF-alpha that also binds to mouse TNF-alpha (**Figure 7A**, **7B**). Flow analysis showed Tregs were decreased similarly between Ipi-DM1 with/out Adalimumab compared hIgGFc control group (**Figures 7C** top panel, 7D). Remarkably, the anti-TNF-alpha partially rescued B cells as shown by percentage difference of B cell marker B220 between Ipi-DM1 and in combination with Adalimumab as well as cell number (**Figures 7C** bottom panel, 7E). Peripheral blood samples stimulated with Iononmycin/PMA increased intracellular cytokines TNF-alpha and IFN-gamma for T cells from Ipi-DM1 treated mice compared to hIgGFc control, while that with Ipi-DM1 in combination with Adalimumab had slight increase but the change was insignificant except for IFN-gamma in CD8 T cells (**Figure 7F**, 7G). In contrast, blocking FAS-L with antibody did not result in B cell rescue (**Figure 7-figure supplement 1**).

Discussion

We have reported that pH-insensitive anti-CTLA-4 antibodies trafficked to, and were degraded in the lysosomes.²⁵ Since lysosomal degradation is needed to release DM1 from the antibody for cytotoxicity, the pH-insensitive Ipilimumab was chosen for preparation of antibody-drug conjugate to be used to impact Treg function. We found that in *Ctla4^{h/h}* mice anti-CTLA-4 ADC, Ipi-DM1, can recapitulate the phenotype of loss of B cells associated clinical deficiency of CTLA-4^{8,9}/recycling partner LBRA¹⁰. Our analysis revealed B cell loss in both bone marrow and peripheral blood, specifically mature B cells. Loss of mature peripheral B cells is consistent with clinical data for some patients with CTLA-4 haploinsufficiency.^{8,9}

B cell loss was previously reported in mice with either naturally occurred (in Scurfy mice) or targeted mutation of *Foxp3⁺* and Treg depletion.^{16,21} The kinetics of B cell loss and Treg depletion in the Ipi-DM1 treated mice revealed that B cell loss was transient and correlated to Treg impairment (proliferation, CTLA-4 level, cell number). Our data further showed that B cell destruction under Treg impairment conditions is T cell-mediated and required T cell activation.

Scurfy mice have poor central and peripheral B lymphopoiesis, however neonatal WT Treg adoptive transfer in scurfy mice resulted in robust population of mature B cells in the spleen.¹⁹ Genetic ablation of *TCRA* gene by crossing scurfy with *TCRA^{α-/-}* mice efficiently support B cell lymphopoiesis and was sufficient to restore B cells in the bone marrow and peripheral compartments.¹⁹ Additionally, reconstitution of bone marrow chimera from scurfy or wild type mixed with that from *μMT* mice that is deficient in B cells results in normal B cells after 8 weeks from reconstitution.¹⁸ This is in line with our data that Treg impairment results in activated T-cell-mediated apoptosis of mature B cells. Mature B cell loss in bone marrow explains the loss of B cell in the PBL and rules out the possibility that the loss of B cell in the periphery is merely a consequence of defective circulation of B cells normally produced in bone marrow. Others have implied that under Treg depletion, activated T cells are targeting interleukin 7 (IL-7) secreting ICAM1+ perivascular stromal cells needed to progress B cell progenitors.²¹ IL-7 is essential for B lymphopoiesis transition from Pro-B cell into Pre-B cell.²⁶ However, our model system is distinct where only mature B cells are depleted, while B cell progenitor precursors, and specifically Pre-B cells are not impacted. The involvement of T cells is demonstrated by T cell depletion and requirement for T cell activation is demonstrated by requirement for B7-CD28 interaction for B

cell loss. Furthermore, we found that anti-TNF- α can partially rescue B-cells from loss imposed by Treg impairment. These data showed that TNF- α is one of the mediators of B cell loss. Additional studies are needed to reveal other mechanism for B cells loss in Treg-defective environment.

While in vitro studies by others showed B cell killing by activated CD25⁺CD4 Tregs, but not by CD25⁻ CD4 T cells,²⁷ this is not the case in vivo where B cell loss is associated with Foxp3⁺ Treg loss or impairment.^{16–21} Furthermore, our current study shows that both CD4 and CD8 T cells contributed to B cell loss. The concept that B cells are actively eliminated by T cells in vivo expands our understanding of T-B cell interaction by showing antagonism rather than just “immunological help” from T cells to B cells.

MATERIALS and METHODS

Experimental animals

C57BL/6 mice that express the CTLA-4 protein with 100% identity to human CTLA-4 protein under the control of endogenous mouse *Ctla4* locus, *Ctla4*^{h/h}, have been previously described.²⁸ All animals used in experiments were 7-9 weeks age (female mice were used unless male mice are indicated). No blinding or randomization was used and mice were fairly distributed into different treatment groups so that initial average weight of each group were similar. All mice were maintained at the Research Animal Facility of the Institute of Human Virology at the University of Maryland Baltimore School of Medicine. All animal studies were approved by the Institutional Animal Care and Use Committee.

Cell culture and treatment

CHO cells that were stably transfected with human CTLA-4 have been reported.²³ CHO cells were grown in DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 10% FBS (Hyclone), 100 units/mL of penicillin and 100 μ g/mL of streptomycin (Gibco). Mice Peripheral blood leukocytes were cultured in RPMI-1640 medium (containing 10% FBS and 2% penicillin/streptomycin). All cell lines were incubated at 37 °C and were maintained in an atmosphere containing 5% CO₂.

Viability assay

Wild type CHO (CHO-WT) or human CTLA-4 expressing CHO (CHO-hCTLA-4) cells were seeded at 1,000 cell/well in a flat 96-well plate at 37 °C for 24 hrs in cell culture incubator. The medium was then replaced with fresh medium containing 1/4 serially diluted vehicle (PBS), Ipilimumab or Ipilimumab-DM1 ADC and cell were incubated at 37 °C for additional 72 hrs. Each treatment group was in duplicate or triplicates. CCK-8 viability dye was then added to each well according to manufacturer's protocol and incubated for another (2-2.5 hrs) at 37 °C. Wells were subsequently read for absorbance at 450nm on Spectramax ID3 Molecular Devices plate reader. In case of testing whether soluble human CTLA-4-Ig or mutant can neutralize Ipilimumab or Ipilimumab-DM1, the same conditions above were used except that Abetacept or Belatacept concentrations were kept constant at 6 μ g/mL. Data is normalized according to the following equation $(\text{treatment}_{\text{OD450}} - \text{background}_{\text{averageOD450}}) / (\text{vehicle}_{\text{averageOD450}} - \text{background}_{\text{average}}) \times 100$.

Cell surface CTLA-4 binding

Freshly trypsinized human CTLA-4 expressing CHO (CHO-hCTLA-4) cells were stained with 1/4 serially dilute anti-CTLA-4 Iplimumab or Iplimumab-DM1 ADC in FACS buffer (2% FBS with 2 mM EDTA) for 30 minutes on ice. Cells were then washed twice with FACS buffer and incubated with anti-human IgG AF488 secondary antibody for 20 minutes on ice. Cells were washed twice with FACS buffer and processed on BD Canto II flow cytometer. In the case of whether soluble human

CTLA-4-Ig or mutant can neutralize Ipilimumab or Ipilimumab-DM1 and prevent them from binding cell to surface CTLA-4 the same conditions above were used except that Abetacept or Belatacept concentrations were kept constant at 6 µg/mL.

Peripheral blood T cell stimulation

Peripheral blood samples 50 µL were treated with ACK Lysis buffer and washed with RPMI-1640 medium. Leukocytes were then stimulated with 1 µg/ml each of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO), ionomycin (Sigma Aldrich, St. Louis, MO) and BD GolgiStop™ (BD Biosciences, cat. 51-2092KZ) and cultured in RPMI-1640 medium (containing 10% FBS and 2% penicillin/streptomycin) for 4 hours at 37°C in 96-well plate. Medium was removed and cells were washed twice with FACS buffer (2% FBS with 2 mM EDTA) followed by surface staining and fix/perm and intracellular staining.

Flow Cytometry

Leukocytes from blood or bone marrow were FACS stained directly or after red blood cell lysis with ACK buffer. Fc Receptor was blocked with anti-FCR clone 2.4G2 at 10 µg/mL in FACS buffer for 10 minutes at room temperature and respective surface staining antibodies cocktails were added to each sample and incubated on ice for additional 20 minutes. Cells were then washed twice with 1xPBS and stained with 1x live/Dead Fixable dye Aqua in 1x PBS for 7 minutes at room temperature. Cells were then washed twice with FACS buffer and fixed with eBioscience™ Fopx3/Transcription Factor Staining Buffer Set for 40 minutes. Samples were either washed twice and resuspended in FACS buffer and processed for flow acquisition or further permeabilized for intracellular staining using perm buffer from same kit (eBioscience™ Fopx3/Transcription Factor Staining Buffer Set). In General, all intracellular staining of Fopx3, Ki67, hCTLA-4, GranzymeB, or cytokines were done overnight at 4 degree. In the case of detecting intracellular cytokines, samples were cultured and simulated according to the above protocol followed by surface then intracellular cytokine staining. Apoptosis staining with Annexin V/Pi was performed according manufacturer protocol after surface staining. Samples were acquired by the BD Canto II Flow cytometer and data were analyzed by Flowjo software.

ELISAs

96-well high-binding polystyrene plates were pre-coated with 50 µL of 1 µg/mL of His-hCTLA-4, or mouse TNF-alpha in coating buffer (0.1M bicarbonate) at 4°C overnight. After washing away the unbound protein/antibody thrice with 0.05% PBST, the plates were blocked with blocking buffer (1% BSA in PBST) for 1hr at room temperature. All primary antibody incubation was done in blocking buffer at 4°C overnight or room temperature for 1 hour. For coated His-hCTLA-4 a given concentration of either Ipilimumab/DM1, or hIgGFc/DM1; For coated mouse TNF-alpha a given concentration of Adalimumab or Ipilimumab negative control were used. Following primary incubation plates were then washed with PBST for four times and incubated with a goat anti-human IgGFc HRP conjugate secondary antibody at 1/20,000 dilution for detection in blocking buffer for 1 hr at room temperature. Plates were then washed 4 times with PBST followed by development with 1-Step™ Ultra TMB-ELISA Substrate Solution for 10 minutes and stopped with 2N sulfuric acid. For Immunoglobulin quantification, an ELISA kit was used following manufacturer protocol. Wells were read at 450nm on Spectramax ID3 Molecular Devices plate reader.

Antibody-drug conjugate preparation

Ipilimumab or hIgGFc were buffer exchanged using prepacked column PD-10 into 1X PBS. Ipilimumab or hIgGFc 5 mg each at concentration 1.5 mg/mL were conjugated with SMCC-DM1(15 eq) in 1x PBS in the presence of 10% DMSO at 37°C under mild shaking conditions for 50 minutes to furnish Ipilimumab-DM1 or hIgGFc-DM1 ADC respectively. Reaction mixture was cleaned up from excess SMCC-DM1 by buffer exchange with a in house ADC buffer at pH 6.5 containing (20

mM Histidine, 8 % sucrose, and Polysorbate 80 0.02%) by Econo-Pac 10DG prepacked column according to manufacturer protocol. Confirmed fraction containing ADC by nano drop were combined and concentrated down to 1 mL using Pierce™ Protein Concentrator PES, (30K for hIgGFc-DM1) or (50K for Ipilimumab-DM1) MWCO according manufacture protocol followed by sterile filtration. ADC concentration was determined using BCA assay according to manufacturer protocol. ADC were diluted to 0.4 – 0.5 mg/mL in 1xPBS and A_{280} & A_{252} were recorded on Nanodrop One. The drug to antibody ratio (DAR) was calculated following previous literature.²⁹

Antibodies and fusion proteins used for in vivo studies

CTLA-4-Ig fusion proteins were synthesized by Sydlabs, Inc (Boston, MA). Recombinant Ipilimumab with amino acid sequence disclosed in WC500109302 was produced by Sydlabs Inc. (Boston, MA). Azide-free human IgG-Fc was purchased from Athens Research and Technology (Athens, GA, USA). Antibody-drug conjugates Ipilimumab-DM1 and hIgGFc-DM1 were prepared from parent antibodies in lab. Depletion antibodies anti-mouse Thy1.2, clone 30H12(BE0066); anti-mouse CD4, clone GK1.5 (BE0003-1); and anti-mouse CD8 α , clone 2.43 (BE00-61) were purchased by Bioxcell Inc. (West Lebanon, NH, USA). Anti-mouse FASL, clone MFL3 (BE00319) was purchased by Bioxcell Inc. (West Lebanon, NH, USA). Anti-TNF- α , Adalimumab, clinical grade Humira™ was purchased from Premium Health Services (Columbia, MD, USA).

Other reagents and material

SMCC-DM1 drug payload with cross linker (Cederlane labs/Cayman Chemical Co, 23926-10) Anti-mouse Fc γ R, clone 2.4G2 (Bioxcell Inc, BE0307). Clodrosome® (Encapsula Nano Sciences, SKU# CLD-8909). Mouse anti-human IgGFc secondary antibody (Invitrogen/Thermo Fisher Scientific, 05-42-00). Goat anti-human IgGFc (HRP) preadsorbed (Abcam, ab98624). Polyhistidine-tagged human CTLA-4 (HIS-hCTLA-4) (Sino Biological Inc, 11159-H08H). Mouse TNF- α (Sino Biological Inc, 50349-MNAE). IgE ELISA Kit (Thermo Fisher Scientific/Invitrogen, 88-50460-86). IgG ELISA Kit (Thermo Fisher Scientific/Invitrogen, 88-50400-86). IgM ELISA Kit (Thermo Fisher Scientific/Invitrogen, 88-50470-86). IgA ELISA Kit (Thermo Fisher Scientific/Invitrogen, 88-50450-86). 123 eBeads™ counting beads flow (Thermo Fisher Scientific/Invitrogen, 01-1234-42). NuPage™ BIS-TRIS gels 4-12% (Thermo Fisher Scientific/Invitrogen, NP0335BOX). NuPage™ MOPS SDS Running Buffer (20X) (Thermo Fisher Scientific/Invitrogen, NP0001). NuPage™ LDS sample buffer (4x) (Thermo Fisher Scientific/Invitrogen, NP0007). Protein Ladder (Fisher Scientific, BP3603500). Sucrose (Sigma Aldrich, S0389-1KG). L-Histidine (Sigma Aldrich, H8000-10G). Dimethyl sulfoxide (DMSO) (Santa Cruz Biotechnology, Sc-258801). Polysorbate 80 (Fisher Scientific, L13315). Buffer exchange prepacked column PD-10 (GE health care/ Cytiva Life Sciences, 17085101). Buffer exchange prepacked column Econo-Pac 10DG (Bio-Rad, 7322010). Cell Counting Kit-8 (CCK-8) (Bimake, B34304). 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, 34028). Gibco™ ACK Lysing buffer (Thermo Fisher scientific/Gibco, A1049201). Live/Dead™ Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific/Life Technologies, L34966). eBioscience™ Foxp3⁺ / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific/Invitrogen, 00-5523-00). Pierce™ Protein Concentrator PES, 30K MWCO, 2-6 mL (Thermo Fisher Scientific/ Pierce, 88521) Pierce™ Protein Concentrators PES, 50K MWCO, 2– 6 mL (Thermo Fisher Scientific/ Pierce, 88538). Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific/Pierce, 23227).

Flow antibodies

eBioscience/ Thermo Fisher Scientific (San Diego, CA): APC-eFlour780 anti-mouse CD45, clone 30-F11 (47-0451-82); eFlour450 anti-mouse CD4, clone GK1.5 (48-0041-82); Pacific Blue anti-mouse CD4, clone RM-4-4 (116008); PE-Cyanine7 anti-mouse CD8 α , clone 53-6.7 (25-0081-82); PE-Cyanine7 anti-mouse CD8 β , clone H35-17.2 (25-0083-82); PerCP-Cy5.5 anti-mouse B220, clone RA3-6B2 (45-0452-82); APC anti-mouse Foxp3, clone FJK-16s (17-5773-82); FITC anti-mouse Ki67, clone SolA15 (11-5698-82); FITC Isotype rat IgG2ak, clone eBR2a (11-4321-82); FITC anti-mouse CD44, clone IM7 (11-0441-85); PerCP-Cy5.5 anti-mouse CD62L, clone MEL-14 (45-0621-82); PE-Cyanine7 anti-mouse B220,

clone RA3-6B2 (25-0452-82); APC anti-mouse IgM, clone II/41 (17-5790-82); eFlour 450 anti-mouse CD21/CD35, clone 4E3 (48-0212-82); AF488 anti-mouse TNF- α , clone MP6-XT22 (53-7321-82); AF488 Isotype rat IgG1k, clone eBRG1 (53-4301-80); APC anti-mouse IFN γ , clone XMG1.2 (17-7311-82); APC Isotype rat IgG1k, clone eBRG1 (17-4301-82); FITC anti-mouse GranzymeB, clone NGZB (11-8898-82); Alexa Fluor 488-conjugated goat anti-human IgG (H+L) cross-adsorbed secondary antibody (A-11013). BioLegend (San Diego, CA); PE anti-human CTLA-4, clone BNI3 (369604); PE Isotype mIgG2ak, clone MPC-173 (400212); PerCP-Cy5.5 anti-mouse IgD, clone 11-26c.21 (405710); BV421 anti-mouse CD21/CD35, clone 7E9 (12342); APC Annexin V Apoptosis Detection Kit with PI (640932);

Statistical analysis

The specific tests used to analyze each set of experiments are indicated in the figure legends. Data were analyzed using a two-tailed t-test to compare between two groups, one-way or two way ANOVA (analysis of variance) for multiple comparisons. In the graphs, y-axis error bars represent S.E.M. or S.D. as indicated. Statistical calculations were performed using GraphPad Prism software (GraphPad Software, San Diego, California). Non-significant [ns], * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Reported CTLA-4 level and absolute cell numbers are normalized by the average value of hIgGfc control group.

Acknowledgements

Funding

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Author contributions

M.M.M performed the study with technical assistance from X.D. M.L., X.W., W.W., and C.A.; M.M.M, Y.L, P.Z, and L.S. designed and/or supervised the research. M.M.M and Y.L. wrote the paper with input from other coauthors.

Competing interests

Y.L. and P.Z are co-founders of OncoC4, Inc. Other authors declare no competing interests.

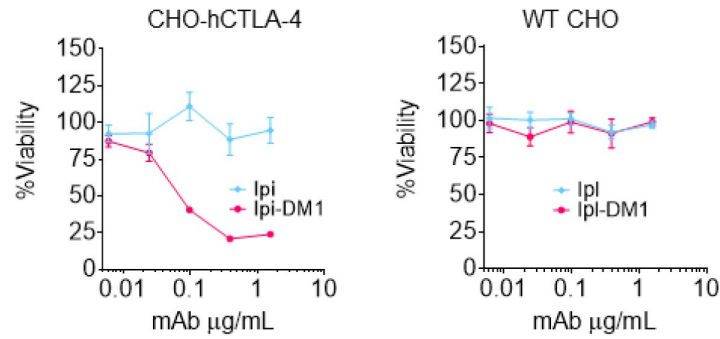


Figure 1-figure supplement 1.

CTLA-4 antibody-drug conjugate killing is CTLA-4 specific.

Cell viability of CHO-hCTLA-4 cells and wild type CHO cells after 72 hours incubation with Ipi or Ipi-DM1 as measured by MTT assay (n=2). Data representative of two or more repeats.

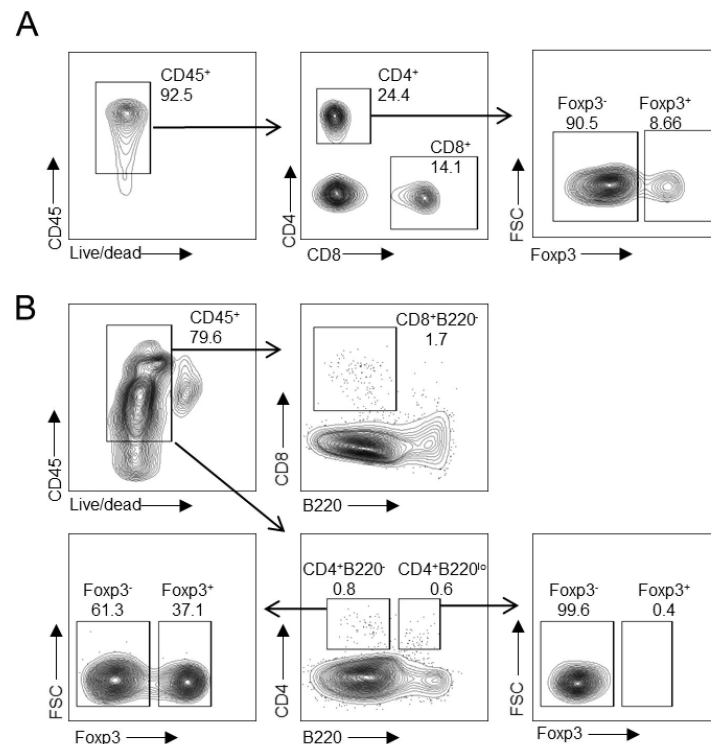


Figure 1-figure supplement 2.

Gating strategy for T cells.

(A) Tregs (CD4⁺ Foxp3⁺) and CD4-nonTreg (CD4⁺ Foxp3⁻) in blood. (B) CD8-T (CD4⁺ B220⁺), CD4-T (CD4⁺ B220⁻), Tregs (CD4 B220 Foxp3) and CD4-nonTreg (CD4⁺B220⁻ Foxp3⁻) in bone marrow.

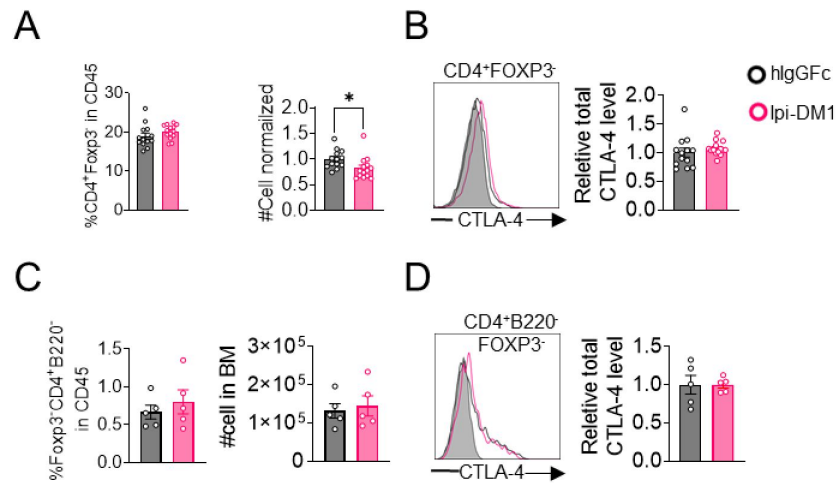


Figure 1-figure supplement 3.

CD4-nonTregs and CTLA-4 expression.

(A, B) Blood, (A) % CD4⁺ Foxp3⁻ in CD45 and normalized cell number, (B) relative CTLA-4 level in CD4-nonTregs. (C, D) Bone marrow, (C) % CD4⁺B220⁻Foxp3⁻ in CD45 and absolute cell number, (B) relative CTLA-4 level in CD4-nonTregs (CD4⁺B220⁻Foxp3⁻). (A, B) Data combined from two independent experiments (n=13-14). (C, D) Data representative of two independent experiments (n=5). Data analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

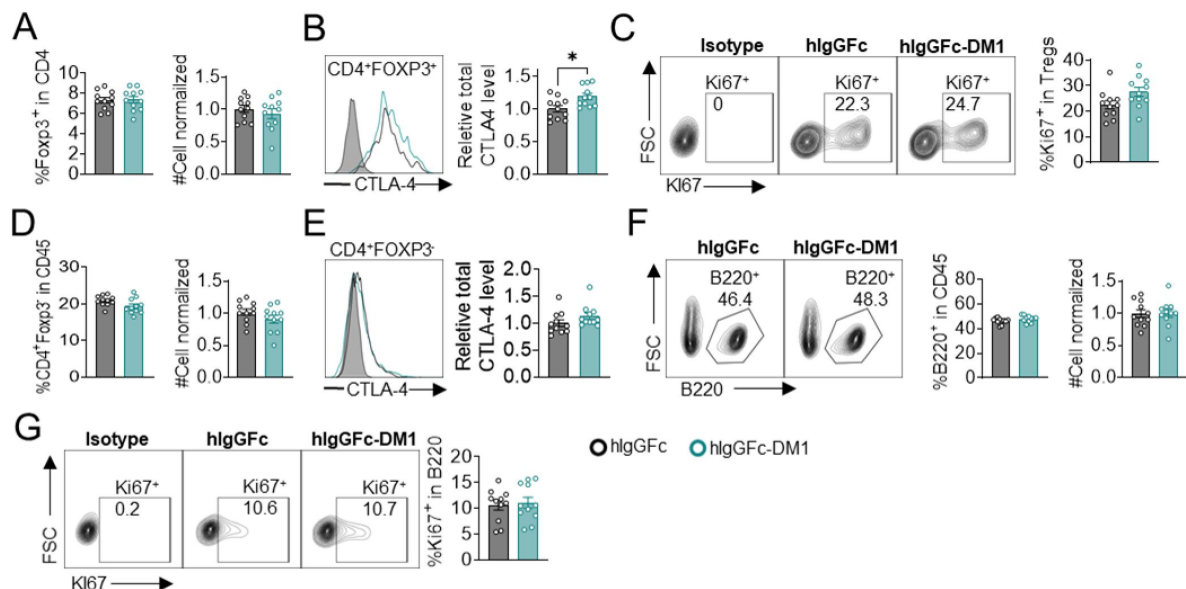


Figure 1-figure supplement 4.

B-cell depletion is not mediated by DM1 payload off-target release.

Mice treated with hlgGFc or hlgGFc-DM1, blood flow analysis on day 9. (A) % Foxp3⁺ in CD4 and normalized cell number. (B) Relative CTLA-4 level in Tregs. (C) % Ki67⁺ in Tregs. (D) % CD4⁺ Foxp3⁻ in CD45 and normalized cell number. (E) Relative CTLA-4 level in CD4-nonTregs. (F) FACS profiles depicting gating strategy after gating on CD45⁺ and data summaries of % B220⁺ in CD45 and normalized cell number. (G) % Ki67⁺ in B cells. Data combined from two independent experiments (n=11) and analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

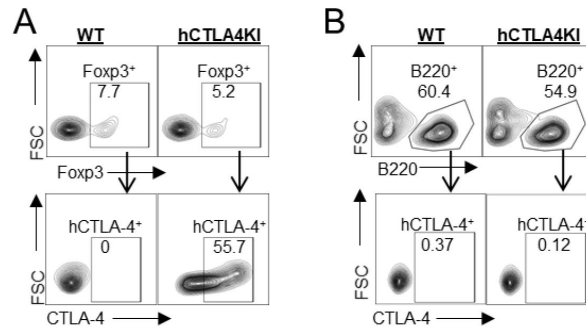


Figure 1-figure supplement 5.

B-cells do not express CTLA-4.

FACS profile of human CTLA-4 in wild-type and human CTLA-4 knock-in mice peripheral blood, (A) % CTLA-4⁺ in Tregs (CD45⁺ CD4⁺ Foxp3⁺), and (B) % CTLA-4⁺ in B cells (CD45⁺ B220⁺). Data representative of 2 mice.

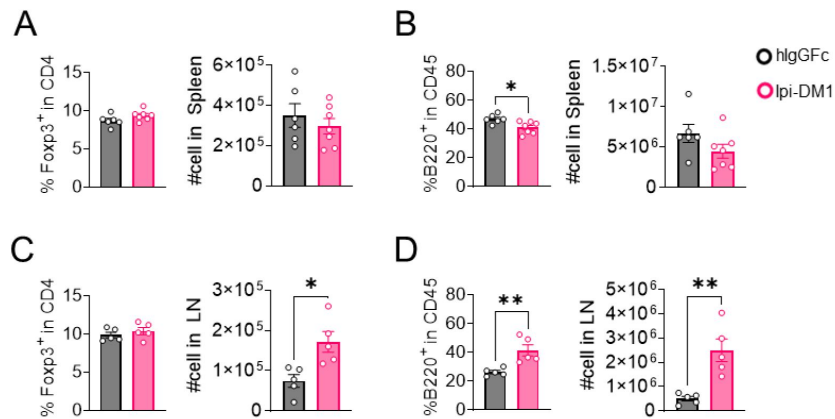


Figure 1-figure supplement 6.

Tregs and B cells in spleen and lymph nodes.

Analysis of spleen and lymph nodes from *Ctl4^{h/h}* mice on day 9 after treatment with hlgGFc or Ipi-DM1 ADC. (A, B) Spleen, (A) % Foxp3⁺ in CD4 and absolute cell number, (B) % B220⁺ in CD45 and absolute cell number. (C, D) combined inguinal and cervical lymph nodes, (C) % Foxp3⁺ in CD4 and absolute cell number, (D) % B220⁺ in CD45 and absolute cell number. Data representative of two independent experiments (n=5-7) and analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. Non-significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

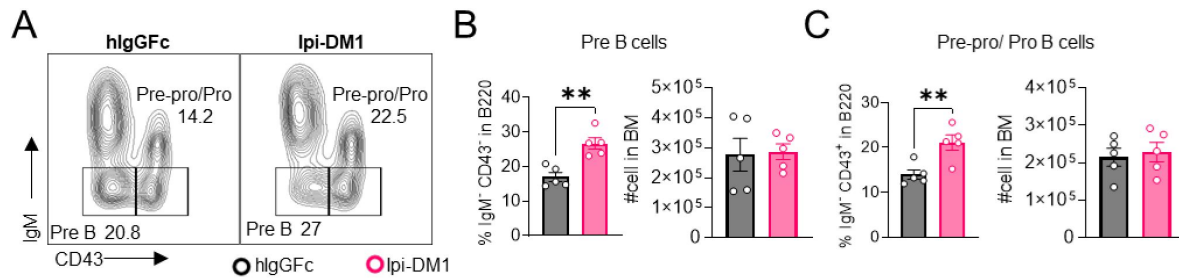


Figure 1-figure supplement 7.

IgM negative B-cell subtypes in bone marrow.

(A) FACS profile gating of Pre B (IgM⁻CD43⁻) and Pre-pro/Pro (IgM⁻CD43⁺) cells in B220. (B-C) % of B cell subtype in B220 and absolute cell numbers, (B) Pre B cells, (C) Pre-pro/Pro B cells. Data representative of two independent experiments (n=5) and analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

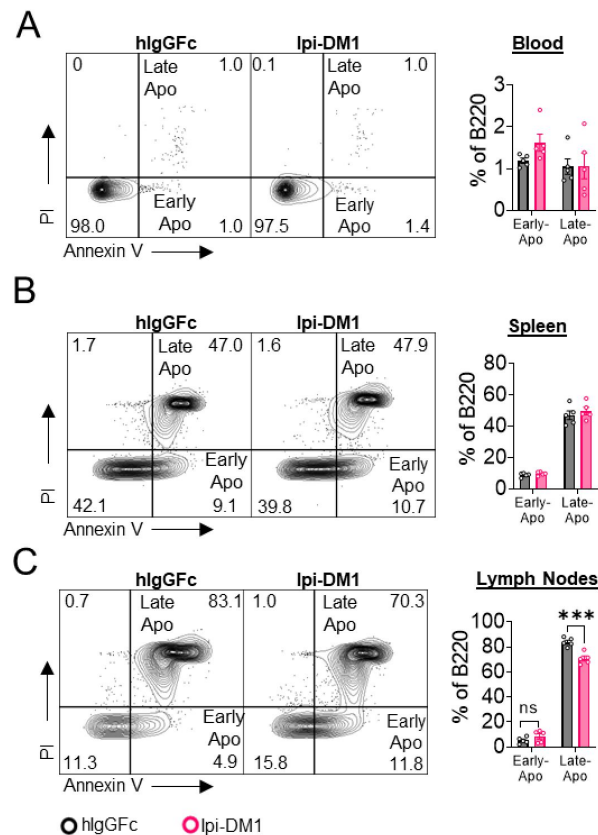


Figure 2-figure supplement 1.

B cell apoptosis in blood and lymphoid organs.

B cell apoptosis on day 9 after treatment with hlgGFc or Ipi-DM1 ADC. (A) B220⁺ blood cells. (B) B220⁺ spleen cells. (C) B220⁺ lymph node cells. Data representative of two independent experiments (n=5) and analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. Non-significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

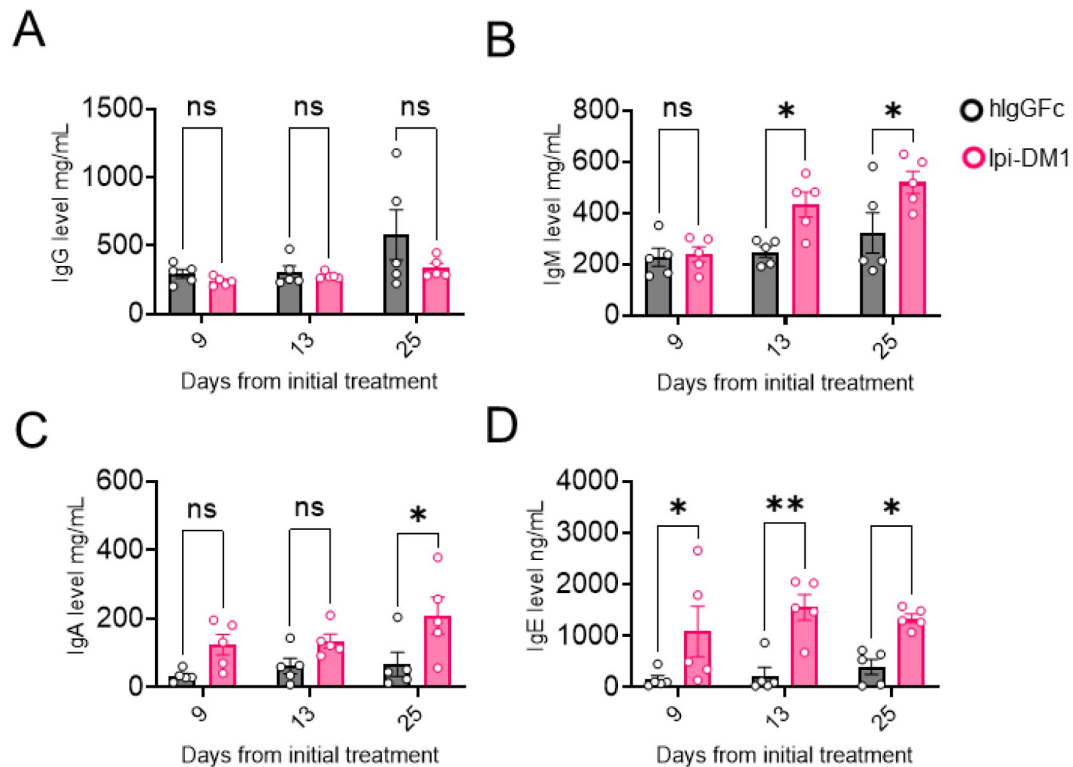


Figure 3-figure supplement 1.

Treg impairment increases plasma immunoglobulins.

Mice treatment with hlgGFc or Ipi-DM1 ADC and plasma collected on day 9, 13, 25 for ELISA Ig level quantification. (A) IgG. (B) IgM. (C) IgA. (D) IgE. Data representative of two independent experiments (n=5) and analyzed using ordinary two-way Anova with Bonferroni's multiple comparisons test and as mean $\pm$ SEM. Non-significant [ns], *p < 0.0332, **p < 0.0021, ***p < 0.0002, ****p < 0.0001.

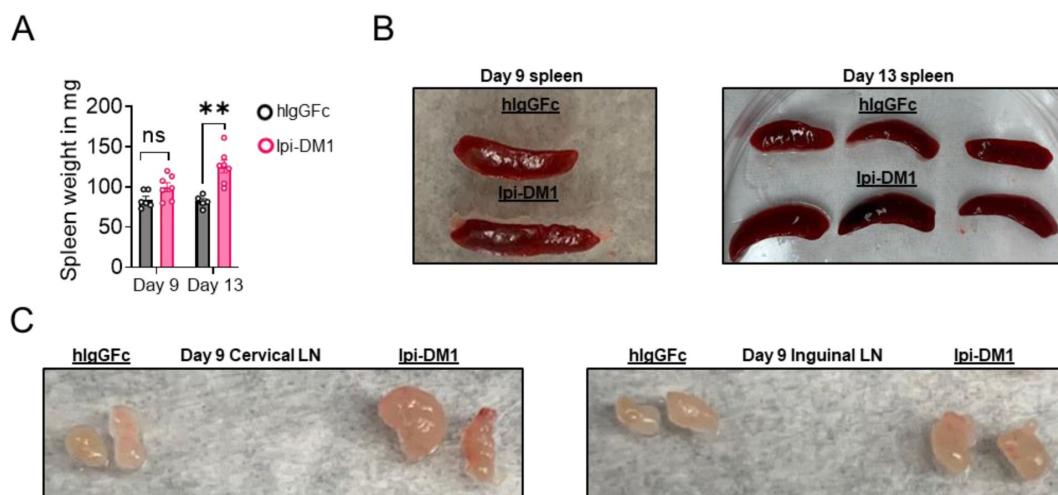


Figure 3-figure supplement 2.

CTLA-4 antibody-drug conjugate leads to enlarged spleen and lymph nodes.

Day 9 or 13 after treatment with hlgGFc or Ipi-DM1 ADC. (A) Spleen weight. (B) Splenomegaly. (C) Lymphadenopathy. (A) Data representative of two independent experiments (n=5-7) and analyzed using an unpaired two-tailed Student's t test and represented as mean $\pm$ SEM. Non-significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

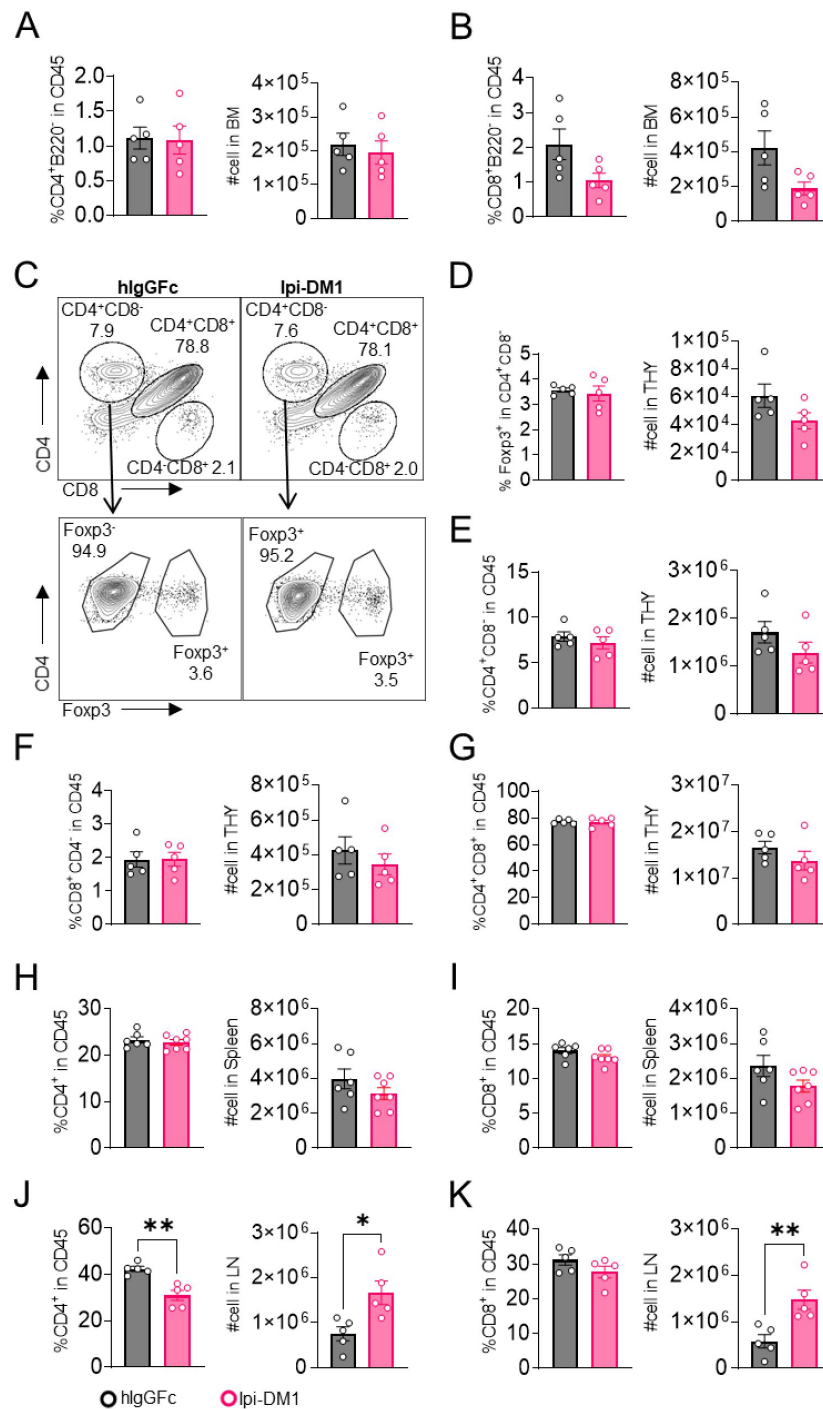


Figure 4-figure supplement 1.

CTLA-4 antibody-drug conjugate impact on T cells in lymphoid organs.

Flow analysis of T cells at day 9 after treatment with hIgGFc or Ipi-DM1 ADC. (A, B) Bone marrow, (C-G) thymus, (H, I) spleen, (J, K) combined inguinal and cervical lymph nodes. (A) % CD4⁺ B220⁻ in CD45 and absolute cell number. (B) % CD8⁺ B220⁻ in CD45 and absolute cell number. (C) Gating strategy defining T-cell types in CD45 cells in thymus. (D) Tregs (% Foxp3⁺ in CD4⁺ CD8⁻) and absolute cell number. (E) % CD4⁺ CD8⁻ in CD45 and absolute cell number. (F) % CD8⁻ CD4⁺ in CD45 and absolute cell number. (G) % CD4⁺ CD8⁻ in CD45 and absolute cell number. (H, J) % CD4⁺ in CD45 and absolute cell number. (I, K) % CD8⁺ in CD45 and absolute cell number. Data representative of two independent experiments (n=5-7) and analyzed using an unpaired two-tailed Student's t test and represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

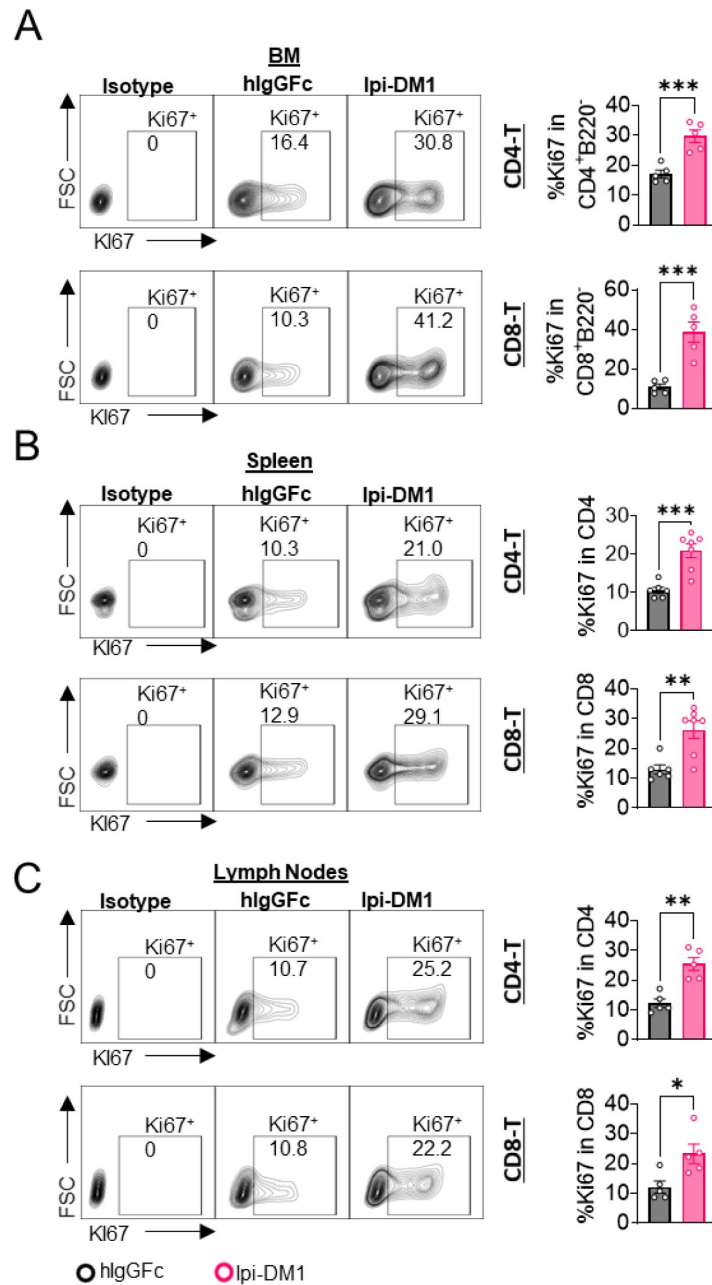


Figure 4-figure supplement 2.

CTLA-4 antibody-drug conjugate increase T cell proliferation in lymphoid organs.

Flow analysis at day 9 after treatment with hlgGFc or Ipi-DM1 ADC. (A) % Ki67⁺ in CD4⁺ B220⁻ and CD8 B220⁻ T cells in bone marrow. (B) % Ki67⁺ in CD4 and CD8 T cells in spleen. (C) % Ki67⁺ in CD4 and CD8 T cells in lymph nodes. Data representative of two independent experiments (n=5-7) and analyzed using an unpaired two-tailed Student's t test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

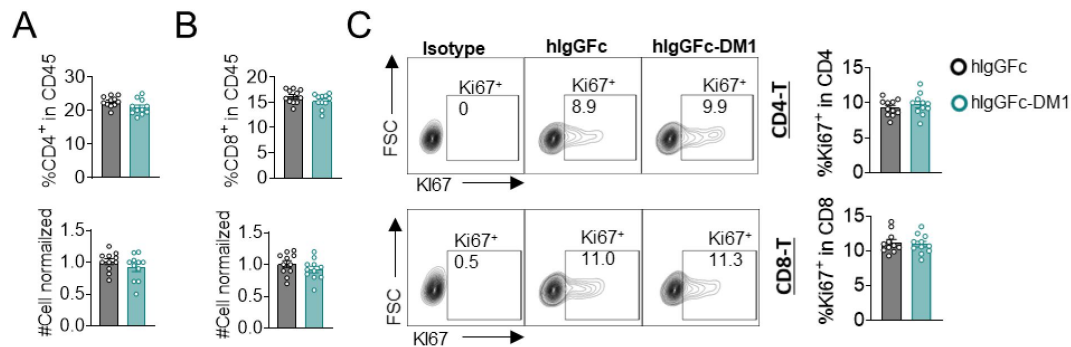


Figure 4-figure supplement 3.

T-cell quantity and proliferation are not impacted by IgG-DM1 treatment.

Mice treated with hlgGFc or hlgGFc-DM1, blood flow analysis on day 9. (A) % CD4⁺ in CD45⁺ and normalized cell number. (B) % CD8 in CD45 and normalized cell number. (C) % Ki67 in CD4 and CD8 T cells. Data combined from two independent experiments (n=11) and analyzed using an unpaired two-tailed Student's t test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

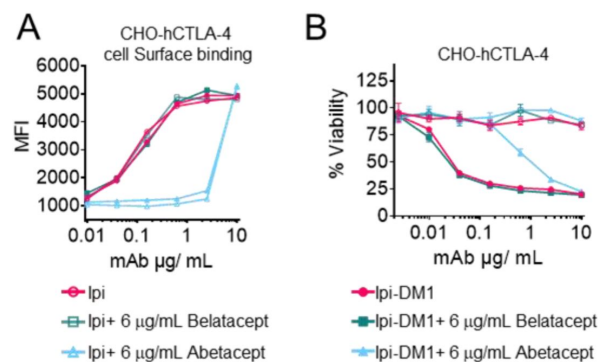


Figure 6-figure supplement 1.

Mutant soluble CTLA-4-Ig does not neutralize Ipilimumab or its drug-conjugate.

(A) Detached CHO cells expressing hCTLA-4 were incubated with serial dilution of Ipi or Ipi-DM1 in the presence of given dose of Abetacept or Belatacept on ice for 30 minutes followed by FACS detection using mouse anti-human IgG AF488, mean fluorescence intensity (MFI). (B) MTT cell viability of CHO-hCTLA-4 cells after 72 hours incubation with Ipi or Ipi-DM1 in the presence of given dose of Abetacept or Belatacept. Data representative of two independent experiments (n=3).

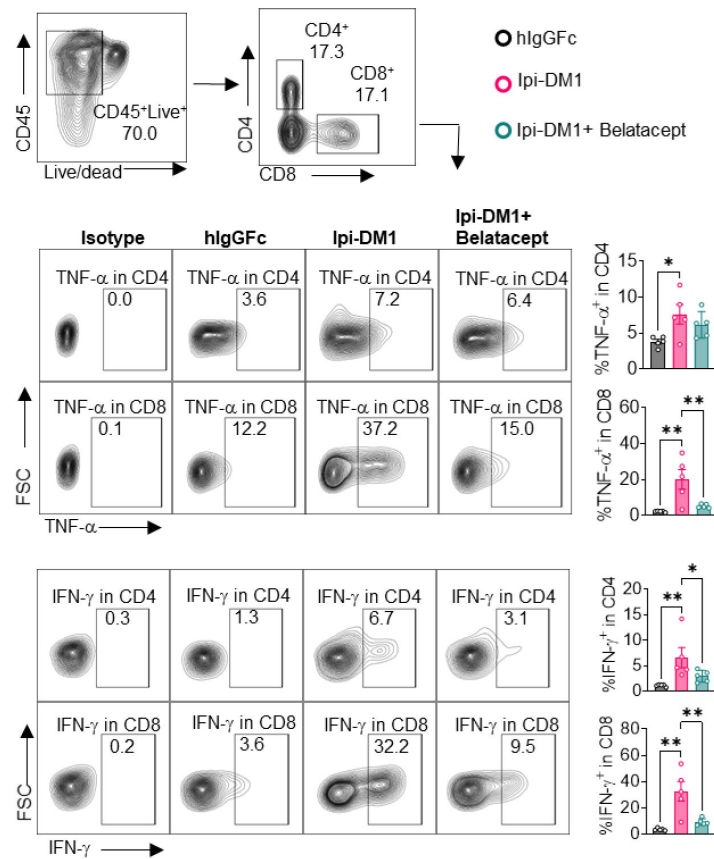


Figure 6-figure supplement 2.

T-cell cytokine production.

Day 13 peripheral blood samples from treated (hlgGFc or Ipi-DM1 with/out Belatacept) mice post ACK buffer red blood lysis were cultured and stimulated in the presence of Iononmycin/PMA, and GolgiPlug for 4 hrs followed by intracellular cytokine detection (TNF-alpha, IFN-gamma) in CD4 and CD8 T cells. Data is representative of two independent experiments (n=5) analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

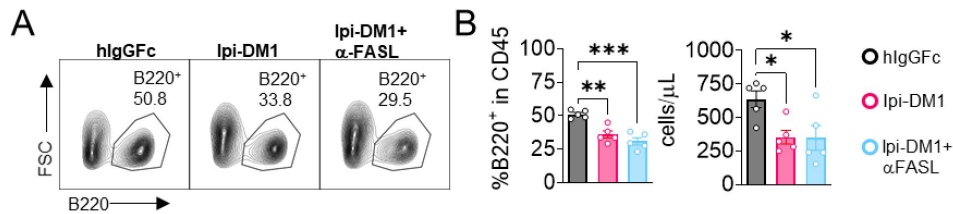


Figure 7-figure supplement 1.

Anti-FASL fails to rescue B cell.

Ctla4^{h/h} mice were treated intraperitoneally (i.p.) (100 μg/mouse) with hlgGFc or Ipi-DM1 with/out (100 μg/mouse of anti-FASL) every three days for a total of three doses and mice were bled on day 9. (A) FACS profile after gating on CD45+ for B cells. (B) % B220 in CD45 and absolute B cell number summaries. Data representative of one experiment (n=5) analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons test and represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Entry	A_{280}	A_{252}	CmAb (Moles)	CDM1(Moles)	DAR CDM1/CmAB
hlgGFC-DM1	0.51	0.48	6.92705E-06	1.1531E-05	1.7
Ipilimumab-DM1	0.62	0.42	2.30582E-06	7.32032E-06	3.2

Drug to antibody ratio (DAR)= CDM1/ CmAB. DM1 known extinction coefficients (A_{280} , 5700 M⁻¹cm⁻¹ ; A_{252} , 28084 M⁻¹cm⁻¹). Experimentally derived extinction coefficients for Ipilimumab (A_{280} , 242115 M⁻¹cm⁻¹ ; A_{252} , 92989 M⁻¹cm⁻¹) and hlgGfc (A_{280} , 64136 M⁻¹cm⁻¹ ; A_{252} , 22544 M⁻¹cm⁻¹)

Table S1.

Drug to antibody ratio (DAR)

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Editors

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Reviewer #1 (Public Review):

The manuscript by Muthana et al. describes the effect of injection of an antibody specific for human CTLA4 conjugated to a cytotoxic molecule (Ipi-DM1) in knock-in mice expressing human CTLA4. The authors show that Ipi-DM1 administration causes a partial decrease (about 50% in absolute number) of mature B cells in blood and bone marrow 9-14 days after the beginning of treatment. B cell progenitors and pre-B cells in the BM are not affected. Ipi-DM1 also results in a partial decrease in Foxp3⁺ Tregs (about 40% in absolute number) and a slight increase in activation of conventional T cells (Tconvs) in the blood, spleen, BM and LNs at D9 as well as increased plasma immunoglobulins especially IgE. Tconv depletion, CTLA4-Ig or anti-TNF mAb partially prevents the effect of ipi-DM1 on B cells. This effect of Ipi-DM1 on the reduction B cells and Tregs at D9 is not observed in the spleen and lymph nodes (maybe not the good timing to see it), and there is even an increase in the number of Treg and the frequency and number of B cells in lymph nodes. This work is interesting but has the following major limitations:

1- This work could have been of more interest if the Ipi-DM1 molecule would be used in the clinic. As this is not the case, the intimate mechanism of the effect of this molecule in mice is of reduced interest.

2- The fact that a partial deletion of Tregs is associated with activation of Tconvs and a decrease in B cells is not new. According to the authors, their work would be the first to show that activation of Tconvs would lead to B cell death. However, this is shown in an indirect way and the mechanisms are not really elucidated. The experiments to try to show a causal link are of 2 types: deletion of T cells (Fig 5) and blocking T cell activation with CTLA4-Ig (Fig 6). These 2 experiments are not fully convincing. The absence of B cell depletion in the blood when T cells are deleted can be explained by other mechanisms, such as B cell recirculation to lymphoid tissues or an effect of massive T cell death for example. The experiment with CTLA4-Ig is more convincing because the effect is targeted to activated T cells only. However,

the prevention of B cell ablation is only partial. Since only blood is analyzed, other mechanisms could explain the B cell loss, such as their recirculation in lymphoid tissues.

3- The authors propose that the drop in B cell numbers in the blood in mice treated with Ipi-DM1 results from reduced mature B cells in the bone marrow. However, B cells are continuously recirculating between the blood and secondary lymphoid tissues. The drop of blood B cells could be well explained by an increased recirculation to lymphoid organs. The increased numbers of B cells in lymph nodes support this latter hypothesis.

4- The new Figure 2 suggests direct evidence of apoptosis of mature B cells in the BM of treated mice using a PI/annexin V staining assay. This is an important point to support the point of the manuscript. However, using the same assay, the level of B cell apoptosis is of 80% in lymph nodes and 50% in the spleen in control mice (see new Figure 2-figure supplement 1), which is way too high and questions the reliability of this assay. It is likely that B cells enter apoptosis only in vitro due to some artefactual stress.

Reviewer #2 (Public Review):

Despite the fact that CTLA-4 is a critical molecule for inhibiting the immune response, surprisingly individuals with heterozygous CTLA-4 mutations exhibit immunodeficiency, presenting with antibody deficiency secondary to B cell loss. Why the loss of a molecule that regulates T cell activation should lead to B cell loss has remained unclear. In this study, Muthana and colleagues use an anti-CTLA-4 antibody drug conjugate (aCTLA-4 ADC) to delete cells expressing high levels of CTLA-4, and show that this leads to a reduction in B cells. The aCTLA-4 ADC is found to delete a subset of Tregs, leading to hyperactivation of T cells that is associated with B cell depletion. Using blocking antibodies, the authors implicate TNF α in the observed B cell loss.

The reciprocal regulation of T and B cell homeostasis is an important research area. While it has been shown that Treg defects are associated with B cell loss, the mechanisms at play are incompletely understood. CTLA-4 is not normally expressed in B cells so an indirect mechanism of action is assumed. The authors show that the decrease in Treg following aCTLA-4 ADC treatment is associated with activation of T cells, and that B cell loss is blunted if T cells are depleted. A role for both CD4 and CD8 T cells is identified by selective CD4/CD8 depletion. T cells appear to require CD28 costimulation in order to mediate B cell loss, since the response is partially inhibited in the presence of the costimulation blockade drug belatacept (CTLA-4-Ig). Finally, experiments using the anti-TNF α antibody adalimumab suggest a potential role for TNF α in the depletion of B cells.

While the manuscript makes a useful contribution, a number of limitations remain. Perhaps most important is the extent to which this model mimics the natural situation in individuals with CTLA-4 mutations (or following CTLA-4-based clinical interventions). aCTLA-4 ADC treatment permits acute deletion of Treg expressing high levels of CTLA-4, whereas in patients the Treg population remains but is specifically impaired in CTLA-4 function. Secondly, although the requirement for T cells to mediate B cell loss is convincingly demonstrated, the incomplete reversal by TNF α blockade suggests additional unidentified factors contribute to this effect. Finally, although the manuscript favours peripheral killing of mature B cells over alterations to B cell lymphopoiesis, one concern is that this may simply reflect the model employed: the short-term (6 day) treatment used here may be too acute to alter B cell development, but this may nevertheless be a feature of prolonged immune dysregulation in humans.

Reviewer #3 (Public Review):

The co-suppressive molecule CTLA-4 has a critical role in the maintenance of peripheral tolerance, primarily by Treg mediated control of the co-stimulatory molecules CD80 and

CD86. As stated by the authors, previous studies have found a variety of effects of anti-CTLA-4 antibody treatment or genetic loss of CTLA-4 on B-cells. These include increased B-cell activation and antibody production, autoantibody production, impairment of B-cell production in the bone marrow and loss of peripheral B-cells. In this article Muthana et al use a CTLA-4 humanized mouse model and examine the effects of drug conjugated CTLA-4 on the immune system. They observe a transient loss of B-cells in the blood of the treated mice. They then use a range of immune interventions such as T-cell depletion and blocking antibodies to demonstrate that this effect is dependent on T-cell activation.

Since anti-CTLA-4 immunotherapy is in active clinical use exploration of its effects are welcome, this is helped by the use of a humanized CTLA-4 system which should be considered a strength of the paper. However, currently the central premise of this paper, that B-cells are depleted seems underexplored. Direct evidence of T-cell killing of B-cells is never presented, rather it is inferred from the reduced numbers of B-cells in the blood and increased apoptosis in the bone marrow. It is not made clear if B-cell numbers in the bone marrow are reduced.

Upon examining lymphoid organs it seems that the spleen is relatively unchanged while the lymph nodes have a large increase in B-cells alongside increased serum antibody levels. The paper does underline the importance of looking at the differences of multiple immune compartments and interesting phenomenon are described in each compartment. Simultaneous inhibition of B-cell lymphopoiesis and blood trafficking with strong activation and antibody production of lymphoid resident (presumably germinal center) B-cells appears to be occurring. However the current overall interpretation that B-cells are broadly depleted is perhaps too simplistic and largely ignores the lymphoid organs and serum antibodies.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

The manuscript by Muthana et al. describes the effect of injection of an antibody specific for human CTLA4 conjugated to a cytotoxic molecule (Ipi-DM1) in knock-in mice expressing human CTLA4. The authors show that Ipi-DM1 administration causes a partial decrease (about 50% in absolute number) of mature B cells in blood and bone marrow 9-14 days after the beginning of treatment. Ipi-DM1 also results in a partial decrease in Foxp3+ Tregs (about 40% in absolute number) and a slight increase in activation of conventional T cells (Tconvs) in the blood at D9. Tconv depletion, CTLA4-Ig or anti-TNF mAb partially prevents the effect of ipi-DM1 on B cells. This work is interesting but has the following major limitations:

- 1. This work could have been of more interest if the Ipi-DM1 molecule would be used in the clinic. As this is not the case, the intimate mechanism of the effect of this molecule in mice is of reduced interest.*

The goal of the current study is to use Ipi-DM1 ADC as probe to study mechanism of B cell loss observed in Treg-deficient host.

1. *The fact that a partial deletion of Tregs is associated with activation of Tconvs and a decrease in B cells has been published several times and is therefore not new. According to the authors, their work would be the first to show that activation of Tconvs would lead to B cell depletion. However, this is shown in an indirect way and the mechanisms are not really elucidated. Indeed, this work shows a correlation between an increase in Tconv activation and a decrease in the number of B cells in the blood. The experiments to try to show a causal link are of 2 types: deletion of T cells (Fig 4) and blocking T cell activation with CTLA4-Ig (Fig 5) (neutralization of TNF addresses another question). Neither of these 2 experiments is totally convincing. Indeed, the absence of B cell depletion when T cells are deleted can be explained by other mechanisms than the preservation of B cell destruction by activated T cells. The phenomenon could be explained by B cell recirculation to lymphoid tissues or an effect of massive T cell death for example. The experiment shown in Fig. 5 with Belatacept is more convincing because this time the effect is targeted to activated T cells only. However, the prevention of B cell ablation is only partial. Again, since only blood is analyzed, other mechanisms could explain the B cell loss, such as their recirculation in lymphoid tissues.*

While the concept of treg depletion leads to activation of Tconv cells and reduced B cells has been previously published, B cell loss was explained on basis of defective B cell lymphopoiesis due to low production of stroma cell-derived IL-7 or destruction of stromal cells by effector T cells. Our new data established that loss of B cells in the context of Treg depletion was not due to defects in the number of pre-/pro-B cells. Rather it is the death of mature B cells in the bone marrow.

To address the reviewer's concern that the B cell loss was merely caused by a change in circulating pattern, we performed a new study on the effect of the ADC on B cells in bone marrow. Our new data reveal loss of mature bone marrow B cells, and that such loss is associated with increased apoptosis of mature B cells. Therefore, the loss of B cells in the peripheral blood is not due to a changed circulation. Furthermore, our data show that B cell progenitor, Pre-B, cells are not changed. Therefore, B cell lymphopoiesis is not the reason for B cell loss in our model system.

1. *It is disappointing that only the blood (and sometimes the bone marrow) was studied in this work. The interest of doing experiments in mice is to have access to many tissues such as the spleen, lymph nodes, colon, lung, and liver. To conclude that there is B cell deletion without showing lymphoid organs (where the majority of B cells reside) is insufficient. As discussed above, the drop in B cells in the blood could be due to their recirculation in lymphoid organs. In addition, there is no measurement of functional B cells activity. Do mice treated with Ipi-DM1 have a decreased ability to develop an antibody response following immunization?*

We have analyzed lymph nodes and spleen at the same time points. Unfortunately, Treg depletion was no longer observed at these time points. As expected, we did not see a clear depletion of B cells (Figure 1-figure supplement 6). In regards to functional B cell activity, we observed an increase of plasma immunoglobulins especially IgE which are now shown in Figure 3-figure supplement 1.

1. *Although it is difficult to study in vivo, there is not a single evidence of increased B cell death after injection of Ipi-DM1.*

Figure 2 & Figure 2-supplement 1 provides B cell death comparisons between IpiDM1 and hIgGfc group for bone marrow, blood, spleen, and lymph nodes. Statistically significant

increase in B cell death is observed in mature B cells in bone marrow.

1. In most of the experiments, B cells are quantified with the B220 marker alone, but this marker, in some cases, can be expressed by other cells. It would have been preferable to use a marker more specific to B cells such as CD19 for example.

We have added data to support the death of mature B cells using other markers.

Minor points.

1. It should be indicated whether human CTLA4 binds normally to mouse CD80 CD86. We do not know if knock-in mice with human CTLA4 have a fully functional immune system.

We have indicated this point as suggested and cited our previous work line 226-227 (ref 23 & 24)

1. The manuscript is too long. Some of the data in the figures should be moved to supplemental figures. This is the case, for example, for some trivial stainings (Fig 1F, Fig 4B, 4F, Fig 5A, D, F, G). The figure legends and the Materials and Methods section are far too long. On the other hand, Fig 5-Fig Sup 1 could go into the main figures.

The figure legends, materials, and methods may be too long, but our intention is to provide as much info as possible for others who may be interested in our model system.

1. The anti-CTLA4 ADC reagent should be better explained and defined in the text.

The anti-CTLA-4 ADC reagent synthesis described in materials/methods under “Antibody-drug conjugate preparation.”

Reviewer #2 (Public Review):

Despite the fact that CTLA-4 is a critical molecule for inhibiting the immune response, surprisingly individuals with heterozygous CTLA-4 mutations exhibit immunodeficiency, presenting with antibody deficiency secondary to B cell loss. Why the loss of a molecule that regulates T cell activation should lead to B cell loss has remained unclear. In this study, Muthana and colleagues use an anti-CTLA-4 antibody drug conjugate (aCTLA-4 ADC) to delete cells expressing high levels of CTLA-4, and show that this leads to a reduction in B cells. The aCTLA-4 ADC is found to delete a subset of Tregs, leading to hyperactivation of T cells that is associated with B cell depletion. Using blocking antibodies, the authors implicate TNFα in the observed B cell loss.

The reciprocal regulation of T and B cell homeostasis is an important research area. While it has been shown that Treg defects are associated with B cell loss, the mechanisms at play are incompletely understood. CTLA-4 is not normally expressed in B cells so an indirect mechanism of action is assumed. The authors show that the decrease in Treg following aCTLA-4 ADC treatment is associated with activation of T cells, and that B cell loss is blunted if T cells are depleted. A role for both CD4 and CD8 T cells is identified by selective CD4/CD8 depletion. T cells appear to require CD28 costimulation in order to mediate B cell loss, since the response is partially inhibited in the presence of the costimulation blockade drug belatacept (CTLA-4-Ig). Finally, experiments using the anti-TNFα antibody adalimumab suggest a potential role for TNFα in the depletion of B cells.

While the manuscript makes a useful contribution, a number of questions remain. Perhaps most important is the extent to which this model mimics the natural situation in individuals with CTLA-4 mutations (or following CTLA-4-based clinical interventions). aCTLA-4 ADC treatment permits acute deletion of Treg expressing high levels of CTLA-4, whereas in patients the Treg population remains but is specifically impaired in CTLA-4 function. Secondly, although the requirement for T cells to mediate B cell loss is convincingly demonstrated, the incomplete reversal by TNF α blockade suggests additional unidentified factors contribute to this effect. Finally, although the manuscript favours peripheral killing of mature B cells over alterations to B cell lymphopoiesis, one concern is that this may simply reflect the model employed: the shortterm (6 day) treatment used here may be too acute to alter B cell development, but this may nevertheless be a feature of prolonged immune dysregulation in humans.

We appreciate reviewer's comments and the difference between short-term depletion and permanent inactivation of Treg by genetic mutation is discussed. We would note that apart from mutation, dynamic Treg perturbation does occur under autoimmune conditions. Therefore, our data have significant implications for T-B cell interactions.

TNF-alpha is implicated in B cell loss as evidenced by the partial rescue with Anti-TNF treatment. We did not try to exclude the possibility that other mechanisms are involved.

Our data shows loss of circulating B cell in peripheral blood and mature bone marrow B cells. B cell progenitor, Pre-B, cells are not changed due Ipi-DM1 induced treg impairment, therefore B cell lymphopoiesis is not the reason for B cell loss in our model system. Evidence of increased cell death is only observed in mature B cells (Figure 2).

1. Following aCTLA-4 ADC treatment, it is surprising how subtle the deletion of Treg is (from ~8% to ~7%, Fig 1G), compared to the marked deletion of CTLA-4-expressing CHO cells. Is this a feature of in vivo versus in vitro treatment? If Treg are treated in vitro is deletion more efficient? How does the expression level of CTLA-4 in the CHO cells compare with the Treg in these assays?

We appreciate reviewer's comments. The anti-CTLA-4 ADC targets CTLA-4 on cell surface. On average about 5% of Tregs express surface CTLA-4 at given moment while human CTLA-4 expressing CHO cell line stains > 90%. Nevertheless, Treg cell number in peripheral blood is reduced by >40%. Additionally, we have included bone marrow data, which shows a greater percentage of Treg depletion (Figure 1J).

1. The decrease in CTLA-4 seen after ipi-DM1 is complicated by the fact that the control DM1 conjugate (IgG1-DM1) appears to significantly increase CTLA-4 expression (Fig 1 supplement 2). It would be useful to clarify when hIgGFc is used versus hIgGFc-DM1 given the additional complexity introduced here (comparisons lacking a payload differ in more than one variable, while the hIgGFc-DM1 is clearly not inert).

We appreciate reviewer's comments. We agree that the hIgGFc-DM1 control slightly increased CTLA-4 level; nevertheless, it did not alter B cells, T cells or their proliferation capacity when compared to hIgGFc. Our point here is that B cell depletion is not mediated by DM1 payload off target release (new-version Figure 1-Figure supplement 4, old version Figure 1-figure supplement 2). As for the clarification comment when hIgGFc is used versus hIgGFcDM1 is used, the information is clarified in the figure legend. Comparisons are made between (hIgGFc VS Ipi-DM1) or (hIgGFc VS hIgGFc-DM1).

1. *T cell-derived IFN γ is another potential contender for influencing B cell homeostasis - have you considered testing whether this also contributes in your model?*

We appreciate reviewer's suggestion. IFN γ was reported to induce apoptosis and cell arrest in Pre- B cells, however these studies are invitro studies Garvey et.al Immunology. 1994 Mar; 81(3): 381–388; Grawunder et.al Eur. J. Immunol. 23, 544–551. Since we did not observe any effect on Pre-B cells, we have not followed the literature to investigate the role of IFN γ in B cell loss in our model.

Reviewer #3 (Public Review):

The co-suppressive molecule CTLA-4 has a critical role in the maintenance of peripheral tolerance, primarily by Treg mediated control of the co-stimulatory molecules CD80 and CD86. As stated by the authors, previous studies have found a variety of effects of anti-CTLA-4 antibody treatment or genetic loss of CTLA-4 on B-cells. These include increased B-cell activation and antibody production, autoantibody production, impairment of B-cell production in the bone marrow and loss of peripheral B-cells. In this article Muthana et al use a CTLA-4 humanized mouse model and examine the effects of drug conjugated CTLA-4 on the immune system. They observe a transient loss of B-cells in the blood of the treated mice. They then use a range of immune interventions such as T-cell depletion and blocking antibodies to demonstrate that this effect is dependent on T-cell activation.

Since anti-CTLA-4 immunotherapy is in active clinical use exploration of its effects are welcome, this is helped by the use of a humanized CTLA-4 system which should be considered a strength of the paper. However, currently, the central premise of this paper, that B-cells are depleted, seems underexplored. Direct evidence of T-cell killing of B-cells is never presented, rather it is inferred from the reduced numbers of B-cells in the blood. The status of B-cells in sites that contain a large proportion of B-cells such as the spleen and lymph nodes is not examined. Additionally, no examination of B-cell antibody production is performed.

We appreciate reviewer's comments. To address the reviewer's concerns we performed additional experiments to evaluate the impact on B cells in other organs, as detailed in our responses to specific questions.

1. *Examination of B-cell apoptosis/cell death and T-cell mediated cytotoxicity is needed. The authors repeatedly refer to auto destructive T-cells without ever demonstrating their presence or any direct evidence that B-cells are dying. This is particularly important in the context of the blood since an alternative hypothesis would be a change in B cell trafficking and infiltration into tissues.*

We appreciate reviewer's comments. To address the reviewer's concern that B cell loss in blood might be caused by a change in B cell trafficking pattern. We performed new study on the effect of the ADC on B cells in bone marrow. Our new data reveal loss of mature bone marrow B cells, and that such loss is associated with increased apoptosis of mature B cells (Figure 2). Therefore, the loss of B cells in the peripheral blood is not due to B cell trafficking and infiltration into tissues.

1. *The authors demonstrate that B-cells are mostly reduced in blood at around days 10 to 15, I believe it is critical to determine if this is also reflected in the lymphoid organs such as the spleen and lymph nodes.*

We appreciate reviewer's comments. We have analyzed lymph node and spleen at the same time points. Unfortunately, Treg depletion was no longer observed at these time points. As expected, we did not see a clear depletion of B cells (Figure 1-figure supplement 6).

1. *Related to the above point do the authors see evidence of Splenomegaly or lymphadenopathy?*

We appreciate reviewer's comment. Evidence of splenomegaly and lymphadenopathy is presented in Figure 3-figure supplement 2.

1. *Minimal examination of the status of the B-cells or antibody production is performed. Previous reports would suggest that plasma cell induction and antibody responses may be expected. Do serum antibody levels change in this system?*

We appreciate reviewer's comment. Increases of plasma immunoglobulins especially IgE are now shown in Figure 3-figure supplement 1.

1. *Its unclear how the authors interpret their experiment with anti-TNFa (figure 6). Are they suggesting that TNFa itself depletes B-cells or that it is part of the inflammatory milieu that contributes to wider T-cell activation and, in turn, B-cell depletion?*

We have discussed these possibilities in the revised manuscript.