Supplemental Materials for:

Depleting T regulatory cells by targeting intracellular Foxp3 with a TCR mimic antibody

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B. **Supplemental Figure 2. (A).** Percentage lymphocyte gate, and numbers after treatment with Foxp3-#32 bispecific mAb. (**B)** Total PBMC numbers after co-cultures with Foxp3-#32 or its control bispecific mAb at 1ug/ml for one to three days. Data show two sets of separate experiments with two donors who are HLA-A*02:01 positive or negative. (C) Absolute numbers of Treg cells after depletion.

C. **Supplemental Figure 3. (A).** Foxp3-#32 bispcific mAb does not deplete Foxp3+ CD4+CD25+CD127low natural Tregs in PBMCs from HLA-A*02:01 negative healthy donor. **(B).** Foxp3-#32 Fc-enhanced human IgG1 depletes Tregs in ascites of HLA-A*02:01 positive patient with ovarian cancer.

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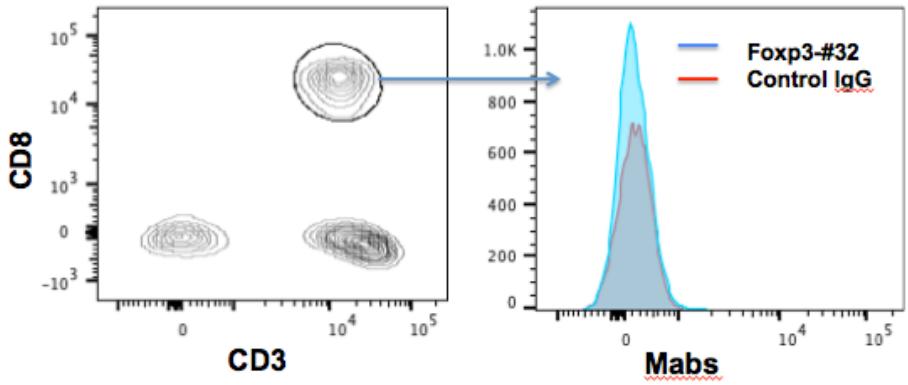
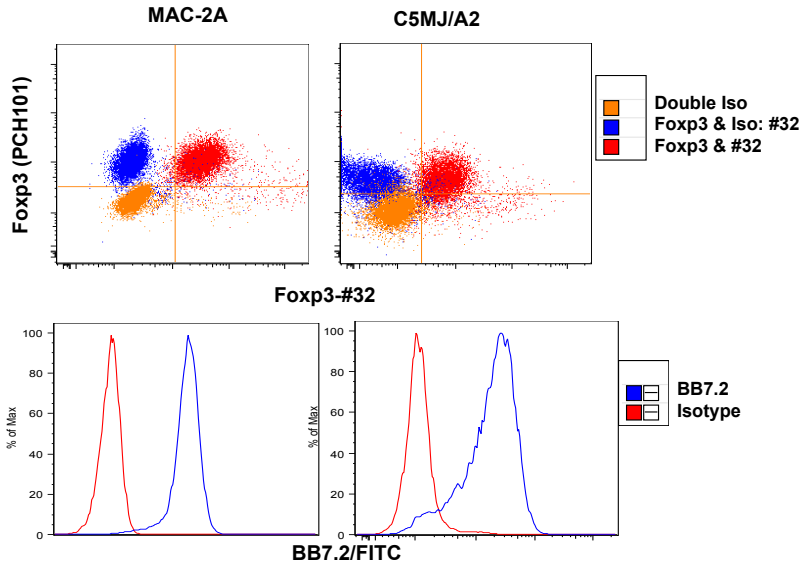
Supplemental Figure 1



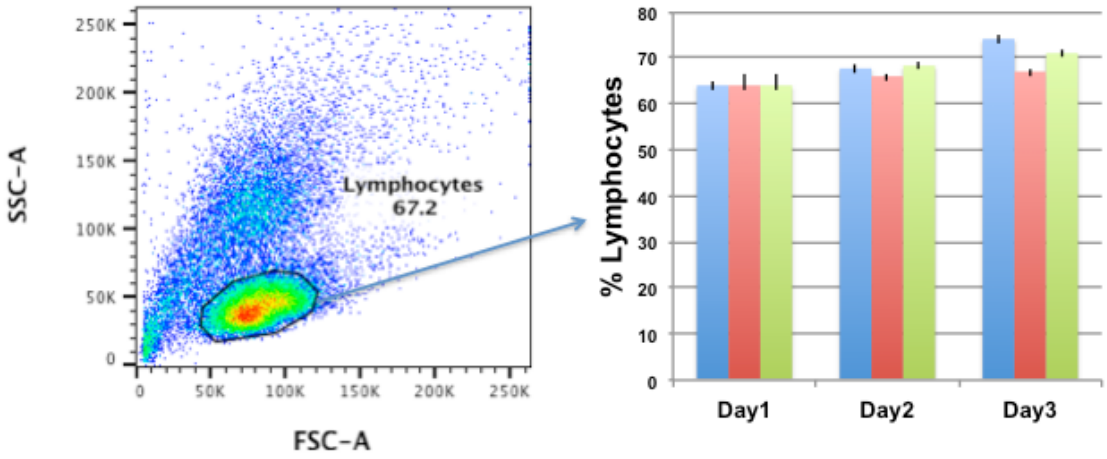
Supplemental Figure 1A. Foxp3-#32 mAb was tested for its binding to CD3/CD8 double positive cells from an HLA-A*02:01 positive healthy donor. No binding was observed compared to control mAb, shown by histogram overlay. Data represent one of flow cytometry data from three donors.



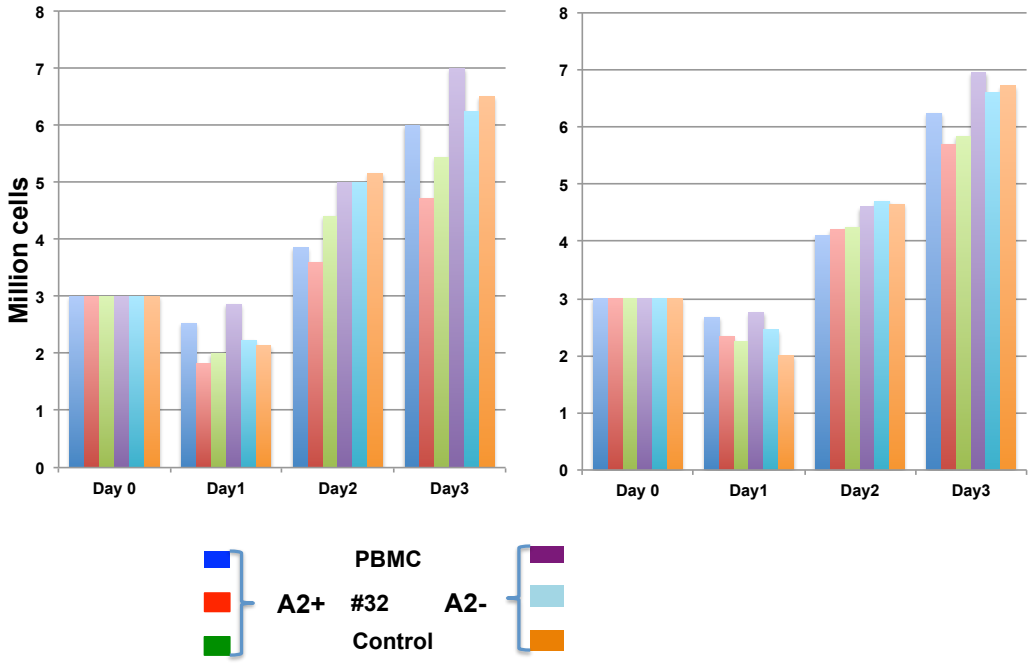
1B. Cell lines MAC-2A and C5MJ transduced with HLA-A*02:01 were stained with mAbs to intracellular Foxp3 vs Foxp3-#32 mouse mAbs. Foxp3-#32 mAb and Foxp3 protein double positive cells were shown in red dots, Foxp3 protein positive cells were not bound by isotype for #32 mAb (blue dots) and isotype controls for both intracellular Foxp3 protein and #32 mAb are shown in orange dots (upper two panels). Histogram shows the HLA-A2 expression in respective cell lines (lower panels). Foxp3-#32 mAb bound only to the cytoplasmic Foxp3+ population (red dots) in both MAC-2A and C5-MJ/A2 cells. In contrast, mouse IgG1 isotype for Foxp3-#32 mAb did not bind to the cytoplasmic Foxp3 protein positive population (blue dots). The results thus show Foxp3-#32 mAb binding to Foxp3+/ HLA-A*02:01 positive cancer cells. However, because we do not have viable A02+/Foxp3 knockout lines available, we cannot determine to what extent the binding to these cancer cell lines is exclusively attributable to the TLI peptide expression, as compared to other possible off-target, cross-reactive peptides.

Supplemental Figure 2.

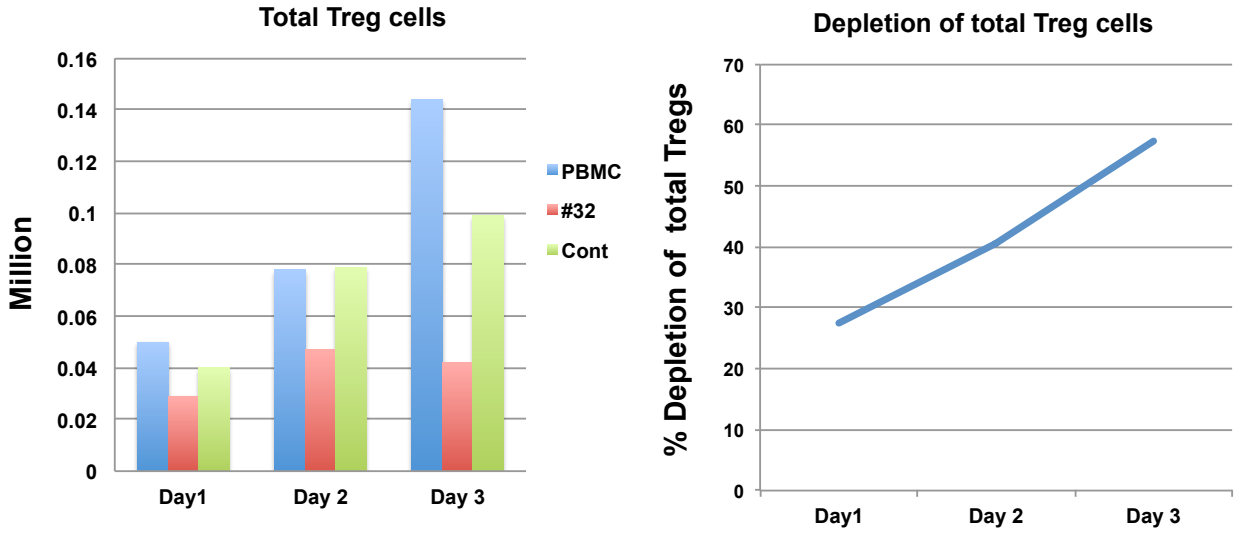
A



Supplemental Figure 2. A. The percentage of lymphocytes remaining was measured by gating on the lymphocyte population in the plot of forward and side scatter (left panel). Representative data show the percentage of lymphocytes numbers in PBMCs from one HLA-A*02:01 positive donor treated with Foxp3-#32 bispecific mAb at 1ug/ml for one, two or three days (right panel, each bar shows triplicate staining plus/minus SD). Blue bar is no mAb; red bar is Foxp3-#32 bispecific mAb treated; green bar is control bispecific mAb treated. A minimal reduction was observed in the Foxp3-#32 bispecific mAb treated group after two and three days of treatment. Data represent one of two similar experiments.

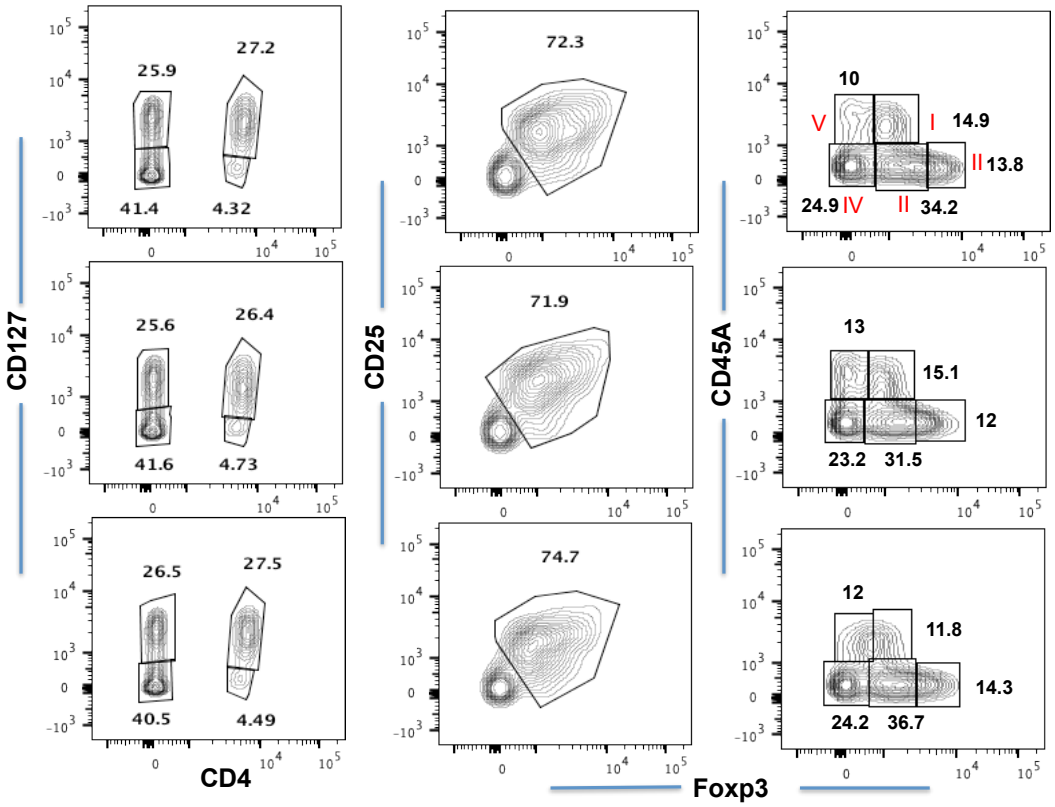


B. Total PBMC during cultures. Three million PBMCs from HLA-A*02:01 positive or negative donor were treated with or withot Foxp3-#32 bispecific or control bispecific mAb (1ug/ml) for three days. Total PBMCs were counted when cells were harvested for flow cytometric analysis from 1 to 3 days using Trypan blue exclusion test. Data show two sets from separate experiments.

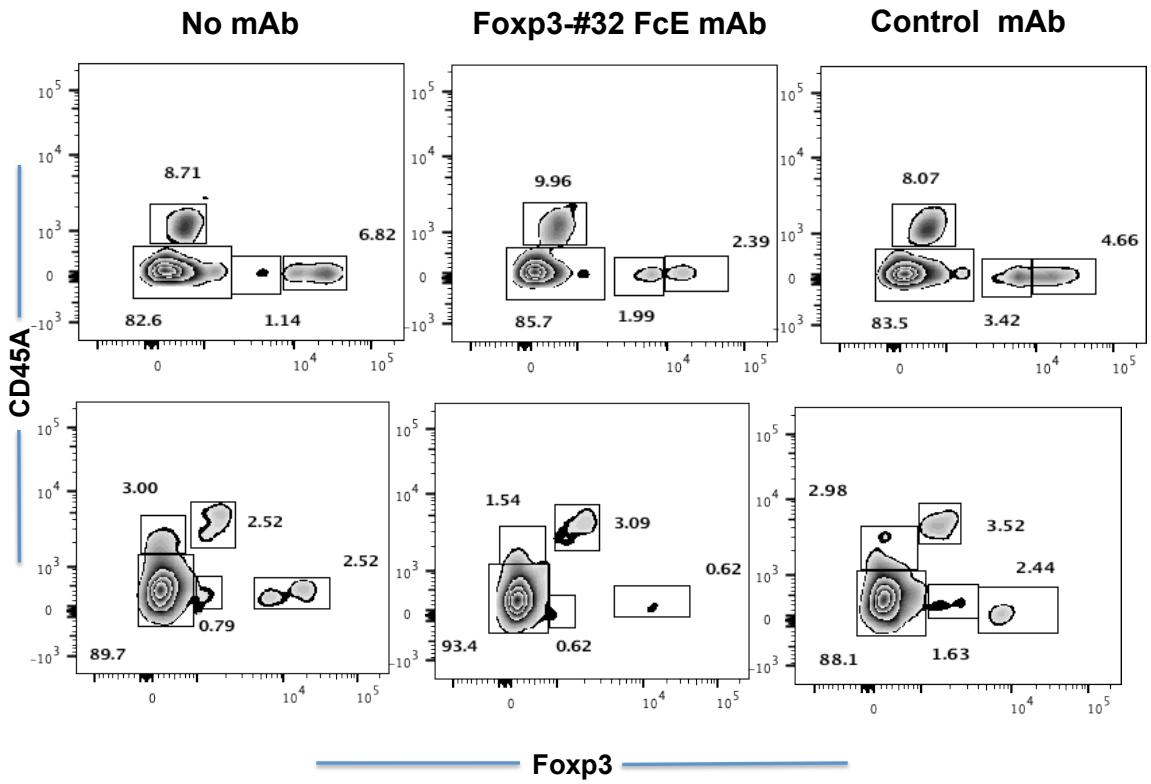


C. Absolute numbers of Treg cells. Total PBMC numbers were multiplied by the percentage of lymphocytes and the percentage of CD4+CD127 low, CD25 high, Foxp3+ populations (Left). Kinetics of total Treg depletion was shown as percentage of absolute numbers of Treg depletion in Foxp3-#32 bispecific mAb over control bispecific mAb group (right). Data show representative data from one of two experiments of two donors who are HLA0A*02:01 positive.

Supplemental Figure 3

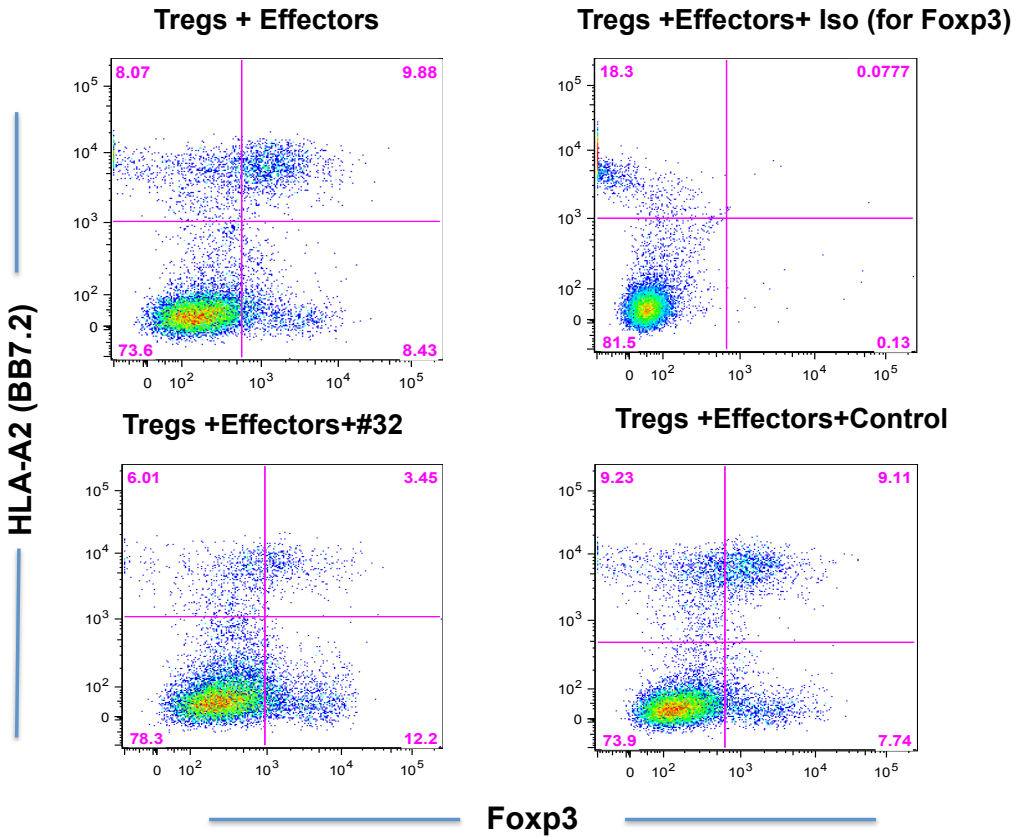


Supplemental Figure 3A. No Foxp3+Tregs were depleted in HLA-A*02:01 negative healthy donor. PBMCs from a healthy HLA-A*02:01 negative donor were treated with Foxp3-#32 bispecific mAb for two days, in the same experiments shown in Figure 7 and Foxp3+ Treg depletion was measured by using Treg markers CD4, CD25, CD127, CD45RA surface staining and Foxp3 intracellular staining. Top three panels: untreated PBMCs; middle three panels: PBMCs treated with Foxp3-#32 bispecific mAb; lower three panels: PBMCs treated with control bispecific mAb. The data show representative data from two similar experiments.

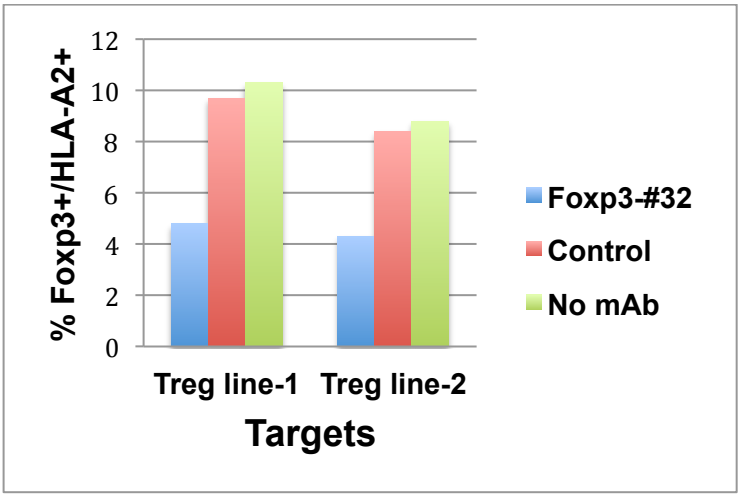


Supplemental Figure 3B. Depletion of Foxp3+Tregs in ascites of a patient with ovarian cancer by Foxp3-#32 Fc-enhanced human IgG1. The ascites cells were treated with Foxp3-#32-Fc-enhanced mAb at a concentration of 10ug/ml for two (upper panels) and three days (lower panels). Representative plot show CD45RA vs Foxp3 staining in the CD4+/CD127low population. Data represent one of two similar experiments.

Supplemental Figure 4

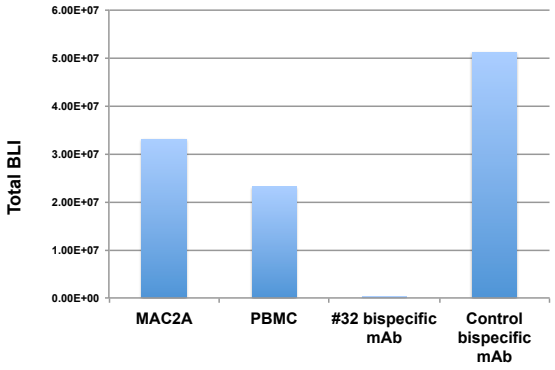


Supplemental Figure 4A. Foxp3-#32 bispecific mAb-mediated T cell killing against *in vitro*-generated Tregs from HLA-A*02:01+ donors. Purified CD3+T cells from a HLA-A2 negative donor were used as effector cells to incubate with Treg lines generated from a HLA-A*02:01+ donor in the presence or absence of Foxp3-#32 or control bispecific mAb (1ug/ml) at an E:T ratio 5:1, overnight. The percentage of Foxp3+ cells in HLA-A*02:01+ T cells was determined by flow cytometry. Reduction of the HLA-A2+Foxp3+ cells indicates the Foxp3-#32 bispecific mAb-mediated T cell killing. Upper left quadrant shows the culture of effector cells alone with Treg line and stained with mAbs to HLA-A2 vs intracellular Foxp3 protein; upper right quadrant shows the culture of effectors with Treg line in the presence of control bispecific mA, but X-axis is the staining with isotype control for intracellular Foxp3 protein to show a specific binding of the mAb to Foxp3 protein in other three panels. Lower two panels show the cultures of effectors with Treg line in the presence of Foxp3-#32- (left) or control bispecific mAb (right). Data show representative flow data from duplicate cultures.

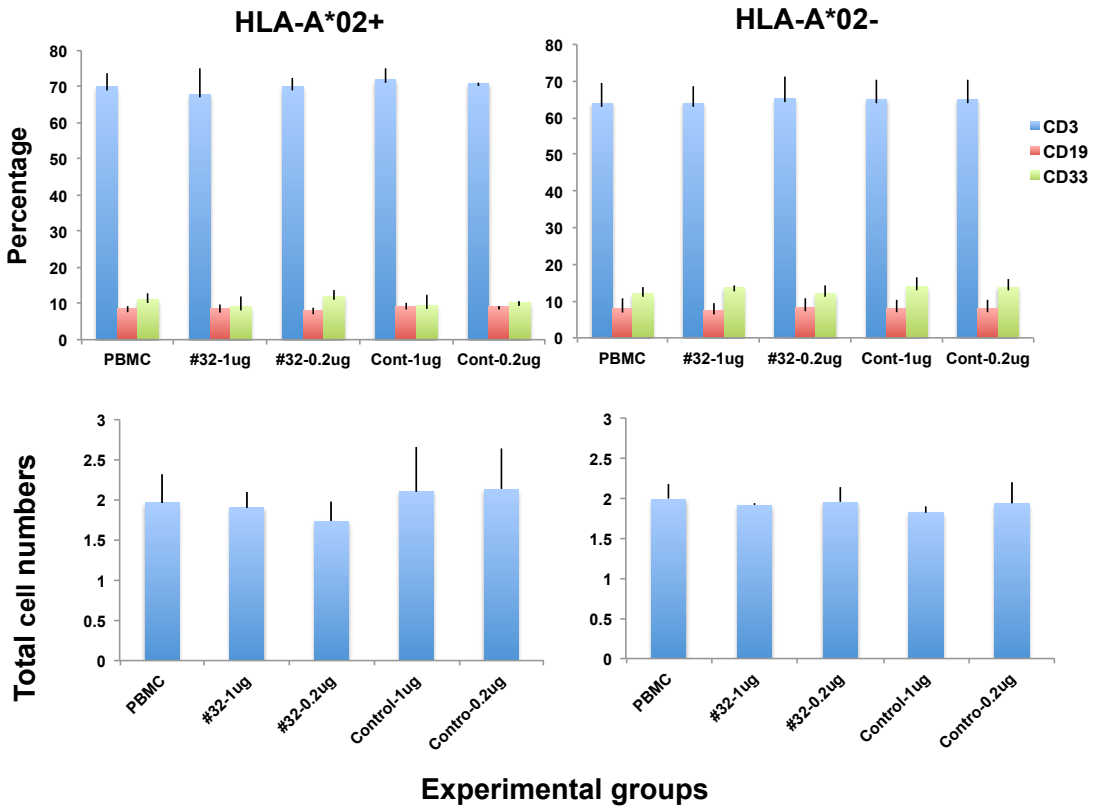


Supplemental Figure 4B. Summary of similar results tested on two Treg lines, as described in supplemental Figure 4A. To further confirm our results, we generated Treg lines from HLA-A*02:01+ donors (phenotype shown in Supplementary Figure 3) and used them as Treg targets. Treg lines used as targets were incubated overnight with purified T cells from HLA-A*02:01-negative donors, in the presence or absence of Foxp3-#32 bispecific mAb or control bispecific mAb. Following this we measured the percentage of Foxp3+ cells in HLA-A*02:01+ T cell population by staining the cells with mAbs to HLA-A2 and intracellular Foxp3 protein. Since HLA-A*02:01+ cells are only present in the target Treg lines, reduction of the HLA-A*02:01and Foxp3 double positive cells indicated Foxp3-#32 bispecific mAb-mediated cytotoxicity against the Tregs (Supplemental Figure 3A). While control cell cultures treated with effector PBMCs alone (upper left panel), or effectors with the control bispecific mAb (lower right panel), showed 9-10% HLA-A*02:01/Foxp3 double positive cells in the co-culture, the percentage of HLA-A*02:01+/Foxp3+ T cells decreased more than 60% in the presence of Foxp3-#32-bispecific mAb (lower left panel). Foxp3+/HLA-A*02:01 negative cells (effector T cells, possibly activated by Treg allo-stimulation) were not killed by the Foxp3-#32 mAb, indicating the HLA-A2 restriction for the mAb recognition. Similar results were obtained from a second Treg line #2 (Supplemental Figure 3B). These results demonstrated that the Foxp3-#32-bispecific mAb wasable to recognize and mediate T cell cytotoxicity against human Tregs in the context of HLA-A*02:01 molecules.

Supplemental Figure 4C. MAC-2A cells that have been transduced with GFP/luciferase was incubated with PBMCs from a HLA-A*02:01 negative donor at an E:T ratio 30:1, in the presence or absence of bispecific mAbs at 1ug/ml for total 3 days. Luciferin 30ug was added to each culture well before imaging. Total bioluminescence was measured in the indicated time points. Data represent average of three microwell cultures.



Supplemental Figure 5.



Supplemental Figure 5. Possible cytotoxicity against normal hematopoietic cells by Foxp3-#32 bispecific mAb. PBMCs from HLA-A*02:01 positive or negative donors were incubated in the presence or absence of 0.2 or 1 ug/ml Foxp3-#32 bispecifc mAb or its isotype control overnight. Cells were washed and stained with mAbs to human CD3, CD19 and CD33 to determine whether these cell lineages are killed by the bispecific mAbs. The percentage of remaining cells in each cell lineage after co-culture is shown (upper two panels) In parallel, total PBMC numbers were counted by Trypan blue exclusion test (lower two panels). Data represent mean+/- SD from three HLA-A*02:01 positive and three HLA- A*02:01 negative donors (*p* values range from 0.3 to 0.6, for groups treated with Foxp3-#32 bispecific mAb or control, as compared to the untreated PBMC groups). In these experiments, as positive controls, MAC-1 cells were incubated with HLA-A*02:01 negative PBMCs as effectors at an E:T ratio of 30:1, in the presence or absence of bispecific mAbs at 1 ug/ml. Cells were harvested and stained with mAb to HLA-A2 (BB7.2 clone). Since only MAC1 cells are HLA-A2 positive, the reduction or disappearance of HLA-A2+ population indicates the killing of MAC-1. All MAC-1 cells were killed under these conditions.

Supplemental Table 1. Sequences of Foxp3-derived peptides

Position	Sequences
p344-353	TLIRWAILEA
p252-260	KLSAMQAHL
p390-398	SLHKCFVRV
p304-312	SLFAVRRHL
p388-396	NLSLHKCFV
p95-103	LLQDRPHFM
p69-77	LQLPTLPLV

Seven human Foxp3-derived peptides were tested for their ability to induce specific T cell response in the context of HLA-A*02:01.

Supplemental Table 2. Absolute numbers of Treg cells

Days after treatment	Total PBMC (million)	% Lymphocy-te	% Tregs : (CD4+CD127^{lo}CD25^{hi}Foxp3+)	Total numbers of Tregs (Thousand)
Day 1				
PBMC	2.5	65.9	66	50
Foxp3-#32 bispecific mAb	1.82	65.5	25.1	29
Control Bispecific	2	65	62.5	40
Day 3				
PBMC	6	74.1	75.9	144
Foxp3-#32 bispecific mAb	4.72	63.6	14.1	42
Control Bispecific	5.44	67.2	71	99

Supplemental Table 2. Cell numbers of PBMCs, percentage of lymphocytes, percentage of CD4+CD127^{lo}CD25^{hi}Foxp3+ cells and absolute Treg counts from a HLA-A*02:01 positive donor, after one and three day cultures with or without Foxp3-32 or its control bispecific mAb. Data show one of two experiments.