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SCIB2, an antibody DNA vaccine encoding NY-ESO-1 epitopes, induces potent antitumor immunity which is further enhanced by checkpoint blockade

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ABSTRACT

Checkpoint blockade has demonstrated promising antitumor responses in approximately 10–40% of patients. However, the majority of patients do not make a productive immune response to their tumors and do not respond to checkpoint blockade. These patients may benefit from an effective vaccine that stimulates high-avidity T cell responses in combination with checkpoint blockade. We have previously shown that incorporating TRP-2 and gp100 epitopes into the CDR regions of a human IgG1 DNA (ImmunoBody[®]: IB) results in significant tumor regression both in animal models and patients. This vaccination strategy is superior to others as it targets antigen to antigen-presenting cells and stimulates high-avidity T cell responses. To broaden the application of this vaccination strategy, 16 NY-ESO-1 epitopes, covering over 80% of HLA phenotypes, were incorporated into the IB (SCIB2). They produced higher frequency and avidity T cell responses than peptide vaccination. These T cells were of sufficient avidity to kill NY-ESO-1-expressing tumor cells, and *in vivo* controlled the growth of established B16-NY-ESO-1 tumors, resulting in long-term survival (35%). When SCIB2 was given in combination with Treg depletion, CTLA-4 blockade or PD-1 blockade, long-term survival from established tumors was significantly enhanced to 56, 67 and 100%, respectively. Translating these responses into the clinic by using a combination of SCIB2 vaccination and checkpoint blockade can only further improve clinical responses.

Abbreviations: Ab, antibody; APC, antigen-presenting cells; CDR, complementary determining region; CTLA-4, cytotoxic T lymphocyte associated protein-4; ELISA, enzyme-linked immunosorbent assay; Elispot, enzyme-linked immunospot; EP, electroporation; FBS, fetal bovine serum; FcR, Fc receptor; IFA/CFA, incomplete/complete Freund's adjuvant; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PI, proliferation index; Treg, regulatory T cells

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Introduction

Checkpoint inhibitors have reformed cancer treatments of melanoma, with 20–30% of patients responding to ipilimumab (CTLA-4 blockade) and to PD1/PD-L1 blockade.^{1,2} More recently, among previously untreated patients with metastatic melanoma, anti-PD-1 alone or combined with anti-CTLA-4 resulted in significantly longer progression-free survival than anti-CTLA-4 alone.³ Although the responses are promising, not all melanoma patients respond and the responses in other cancers have not been as high. Recent studies show that the response to checkpoint blockade may be related to the frequency of neo-epitopes that stimulate new T cell responses that have not been tolerized.^{4,5} However, the majority of patients are non-responders and do not have the appropriate mutations, so they fail to stimulate a productive immune response. These patients may benefit from an effective vaccine that stimulates high-avidity CD4⁺ and CD8⁺ responses in combination with

checkpoint blockade. This is not a new concept as the initial trials with ipilimumab used gp100 peptide vaccination. However, the problem with peptide vaccinations is that they do not generate T cells with sufficient avidity to clear the tumor.⁶

Due to its restricted normal expression and widespread tumor expression (including esophageal, lung, liver, ovarian, melanoma, bladder, prostate and breast cancer),^{7–9} NY-ESO-1 is an ideal target for a tumor therapy. Indeed, T cell responses restricted through multiple HLA alleles have been reported,^{10–12} allowing a cancer vaccine that targets this antigen to be applicable to both a broad range of patients with different HLA types and also to a wide range of tumors. A variety of vaccination approaches have been tried with NY-ESO-1, including administration of synthetic peptide, recombinant protein and DNA encoding full-length NY-ESO-1^{13–15} but they have failed to control tumor growth.^{13,16,17} This may be related to induction of low-avidity T cell responses with restricted ability to recognize

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tumor cells and the profoundly immunosuppressive tumor environment which further restricts T cell activity.

We have previously shown that a DNA plasmid encoding T cell epitopes within the complementary determining regions (CDR)¹⁸ of a human IgG1 antibody (ImmunoBody[®]) stimulates high-avidity T cell responses.¹⁹ Electroporation (EP) increases DNA uptake over 1,000-fold and has an adjuvant effect resulting from local tissue damage and the subsequent expression of pro-inflammatory cytokines.^{20,21} The ImmunoBody[®] acts by direct presentation of the DNA within antigen-presenting cells and cross-presentation of secreted protein via the high-affinity FcγR1 receptor (CD64). When comparing DNA and protein immunization of ImmunoBody[®], the DNA gave higher frequency and avidity responses suggesting direct presentation of the DNA within antigen-presenting cells. However, experiments in CD64-knockout mice but not CD32-knockout mice induced lower frequency and avidity T responses in wild-type mice suggesting that cross-presentation of secreted protein via the high-affinity FcγR1 receptor (CD64) was important. Although either presentation induces T cell responses, it is only the combination that induces T cells with sufficiently high avidity to kill tumor cells.^{18,19} This was further validated by comparison of the same ImmunoBody[®] DNA expressing Fab or whole antibody molecules, which showed much weaker responses in the absence of Fc. We have also replaced human IgG1 from the same DNA backbone vector with mIgG2a; both huIgG1 and mIgG2a can stimulate immune responses in mice.¹⁷ SCIB1 is an ImmunoBody[®] encoding a human IgG1 antibody, with three epitopes from gp100 and one from TRP-2 engineered into its CDR regions, which has entered clinical trials. A clinical study in stage III/IV melanoma patients with tumor present at study entry showed that SCIB1 could induce T cell responses in 10/11 patients with no associated toxicity. Overall survival was 19 mo with patients showing clinical responses including two partial responses and stable disease. Results were even more dramatic in patients with fully resected disease as they all showed a T cell response and are still alive with a current median observation time of 3 y. Two- and three-year recurrence-free survival for stage III resected patients was 89 and 67%, respectively, and for stage IV resected patients, it was 71% at both time points.²²

In this study, we report the validation of SCIB2 which encodes NY-ESO-1 T cell epitopes in the CDR regions of the human IgG1 vector. This approach stimulates higher avidity T cell responses than peptide and demonstrates the advantage of combining both high-avidity T cell responses with checkpoint blockade. The combination of SCIB2 with PD1 blockade led to complete tumor regression.

Results

Melanoma patients demonstrate NY-ESO-1-specific T cell responses

Previous studies suggest NY-ESO-1-specific T cell responses could be detected in peripheral blood mononuclear cells (PBMCs) from cancer patients.²³ We selected 16 epitopes from NY-ESO-1 covering the most common class I and class II haplotypes and confirmed that melanoma patients show T cell responses to these peptides. 10 of 11 (90%) patients responded to one or more of the CD8⁺ epitopes (Fig. 1A) and 9 of 11

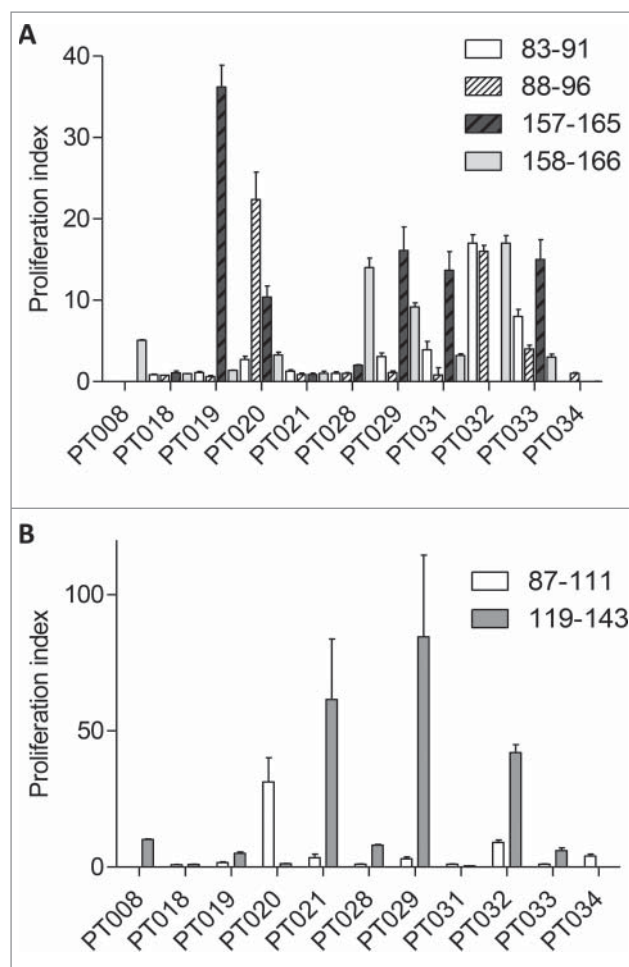


Figure 1. Peripheral blood lymphocytes (PBMC) from melanoma patients show spontaneous response to selected NY-ESO-1 peptides. The *in vitro* proliferation assay was performed on PBMC from melanoma patients following 10 d incubation with NY-ESO-1 (A) CD8⁺ peptides (NY-ESO-1 83–91, 88–96, 157–165, 158–166) and (B) CD4⁺ peptides (NY-ESO-1 87–111 and 119–143).

(82%) to one or more of the CD4⁺ epitopes (Fig. 1B). The clinicopathology and HLA types are shown in Table S1. This data suggested that inclusion of these epitopes within SCIB2 would provide a vaccine that could target the majority of HLA types. Table 1 summarizes the HLA-restricted NY-ESO-1 epitopes that have been incorporated into SCIB2.

Immunization with SCIB2 generates strong NY-ESO-1-specific CD8⁺ and CD4⁺ responses in HLA transgenic mice

HHDII (HLA-A*0201) transgenic mice immunized with SCIB2 showed high-frequency responses to the CD8⁺ T cell epitope NY-ESO-1 157–165, and the CD4⁺ T cell epitopes 87–111 and 119–143 over background control (Fig. 2A) confirmed by *in vitro* CD8⁺ and CD4⁺ depletion, respectively (Figs. S1A and B). In this instance, the CD4⁺-mediated responses were I-Ab restricted (Fig. S1B).

To assess the immune response in a mouse with only human MHC, we immunized HHDII/DR1 mice that have human class I HLA-A*0201 and human class II (HLA-DR1) and no mouse MHC. As illustrated in Fig. 2B, T cells from immunized HHDII/DR1 mice show significantly higher epitope-specific responses to NY-ESO-1 157–165 over background control. This is consistent

Table 1. NY-ESO-1 incorporated epitopes.

Site	Epitope coordinates	Epitope sequence	HLA	HLA frequency (%)	References
H1	158–166	LLMWITQCF	A24	41	11
H2	157–165	SLLMWITQC	A2	44	52–54
H3	83–111	PESRLLEFY LAMPFATPMEAE LARRSLAQ			Patents WO 2001007917 A1
	83–91	PESRLLEFY	B44	21	55
	86–99	RLLEFY LAMPFATP	DR2	25	56
	88–96	LEFY LAMPF	B18	6	55
	89–100	EFY LAMPFATPM	DR1	18	57,58
	94–102	MPFATPMEA	B35/B51	20/12	59
	92–100	LAMPFATPM	Cw3	17	60
	87–100	LLEFY LAMPFATPM	DR9	3	12
	87–111	LLEFY LAMPFATPMEAE LARRSLAQ	DR1/4/7/DP4	18/24/25/75	10,25,26,61
L1	119–143	PGVLLKEFTVSGNILTIRLTAAADHR	DR1/4/7	18/24/25	24
	123–137	LKEFTVSGNILTIRL	DR52b	25	60
	124–134	KEFTVSGNILT	DR8	4	62
	124–135	KEFTVSGNILT	B49	1	63
	127–136	TVSGNILTIR	A68	8	63
	127–135	TVSGNILT	Cw15	3	63

with Fig. S1C showing paired response between background control and NY-ESO-1 157–165. SCIB2-immunized mice also showed significantly higher antigen-specific responses to 87–111 and 119–143, indicating that the 87–111 and 119–143 sequences also induce responses restricted through HLA-DR1 (Fig. 2B). Addition of HLA-DR-blocking Ab into the assay confirmed that these responses were HLA-DR-restricted CD4⁺ responses (Fig. S1D). To assess if the DNA vector alone could act as an adjuvant and generate NY-ESO-1-specific immune responses, mice were immunized with vector expressing the human IgG1 antibody with no NY-ESO-1 epitopes inserted. The empty antibody vector did not generate any NY-ESO-1-specific IFN γ responses (Fig. S1E). In addition, no responses to irrelevant peptides were observed in SCIB2-immunized mice (Fig. S1F).

High-avidity T cell (3.8×10^{-9} , 1.7×10^{-8}) responses were demonstrated by titration of the NY-ESO-1 157–165 peptide in both HHDII and HHDII/DR1 mice (Fig. 2C). These high-avidity T cell responses result in killing of NY-ESO-1-positive tumor cells (B16/HHDII/NY-ESO-1 cells) but not of HLA-mismatched or antigen-negative control cells (Fig. 2D). This data demonstrated that SCIB2 can be used *in vivo* to stimulate strong CD8⁺ T cell responses that are capable of tumor cell lysis *in vitro* as well as induction of CD4⁺ T cell responses.

To further assess the cytotoxic potential of the encoded epitopes, splenocytes from mice immunized with SCIB2 were incubated with NY-ESO-1 peptides *in vitro* for 40 h and the supernatants were analyzed by granzyme B ELISA. All three epitopes stimulated significant amounts of granzyme B including the CD4⁺ epitopes NY-ESO-1 87–111 and 119–143 epitopes, these responses could be completely blocked by mouse MHC class II blocking Ab (Fig. 2E).

SCIB2 induces higher avidity CD8⁺ responses than peptide vaccination

Many clinical trials using NY-ESO-1 vaccines have failed to show clinical benefits in patients. To determine whether SCIB2 was likely to be more potent, the frequency and avidity of T cell responses generated from vaccination with SCIB2 and conventional peptide immunization were compared. SCIB2 immunization stimulated higher frequency T cell responses specific for the NY-ESO-1 157–

165 epitope than peptide immunization (SCIB2 vs. peptide $p = 0.0004$) (Fig. 3A). The functional avidity, as measured by peptide titration, showed that SCIB2 (9×10^{-9} M) generated a 100-fold higher avidity than peptide (10^{-6} M) immunized mice (Fig. 3B).

Differences in functional avidity have been directly correlated with antitumor activity. NY-ESO-1 157–165-specific T cells induced by immunization with either SCIB2 or with peptide were able to specifically recognize peptide pulsed tumor targets; however, SCIB2 immunization was the only approach which showed recognition and lysis of naturally processed and presented NY-ESO-1 antigen on tumor cells (Fig. 3C). Overall, this demonstrated that NY-ESO-1 157–165-specific T cells generated from mice immunized with SCIB2 were more efficient at targeting tumor cells *in vitro*.

Immunization with SCIB2 generates strong antitumor immunity

To see if the enhanced *in vitro* recognition of tumor targets was also reflected *in vivo*, mice with established B16/HHDII/NY-ESO-1 tumors were immunized with SCIB2. Mice immunized with SCIB2 demonstrate a significant delay in tumor growth ($p < 0.05$) (Figs. 4A, B and C) and 35% mice remain tumor-free with long-term survival ($p = 0.0001$) (Fig. 4D).

T cell responses induced by combining SCIB2 with anti-CD25 Ab or checkpoint blockade

Results from clinical studies have suggested that Treg depletion or checkpoint blockade can enhance endogenous T cell responses and/or recover immune responses within the tumor environment. Initially, the effect of Treg depletion or checkpoint blockade on SCIB2 immune responses was assessed. We have shown that anti-CD25 Ab could effectively eliminate Tregs *in vivo*. The percentage of CD4⁺CD25⁺ FOXP3⁺ cells was reduced from 0.53% of CD4⁺ cells to 0.00% after administration of anti-CD25 Ab. However, there was also a depletion of CD4⁺CD25⁺ cells from 1.78 to 0.13% suggesting some activated CD4⁺ cells may also have been depleted. Therefore, prior to the antitumor studies, it was important to assess the effect of anti-CD25 Ab on

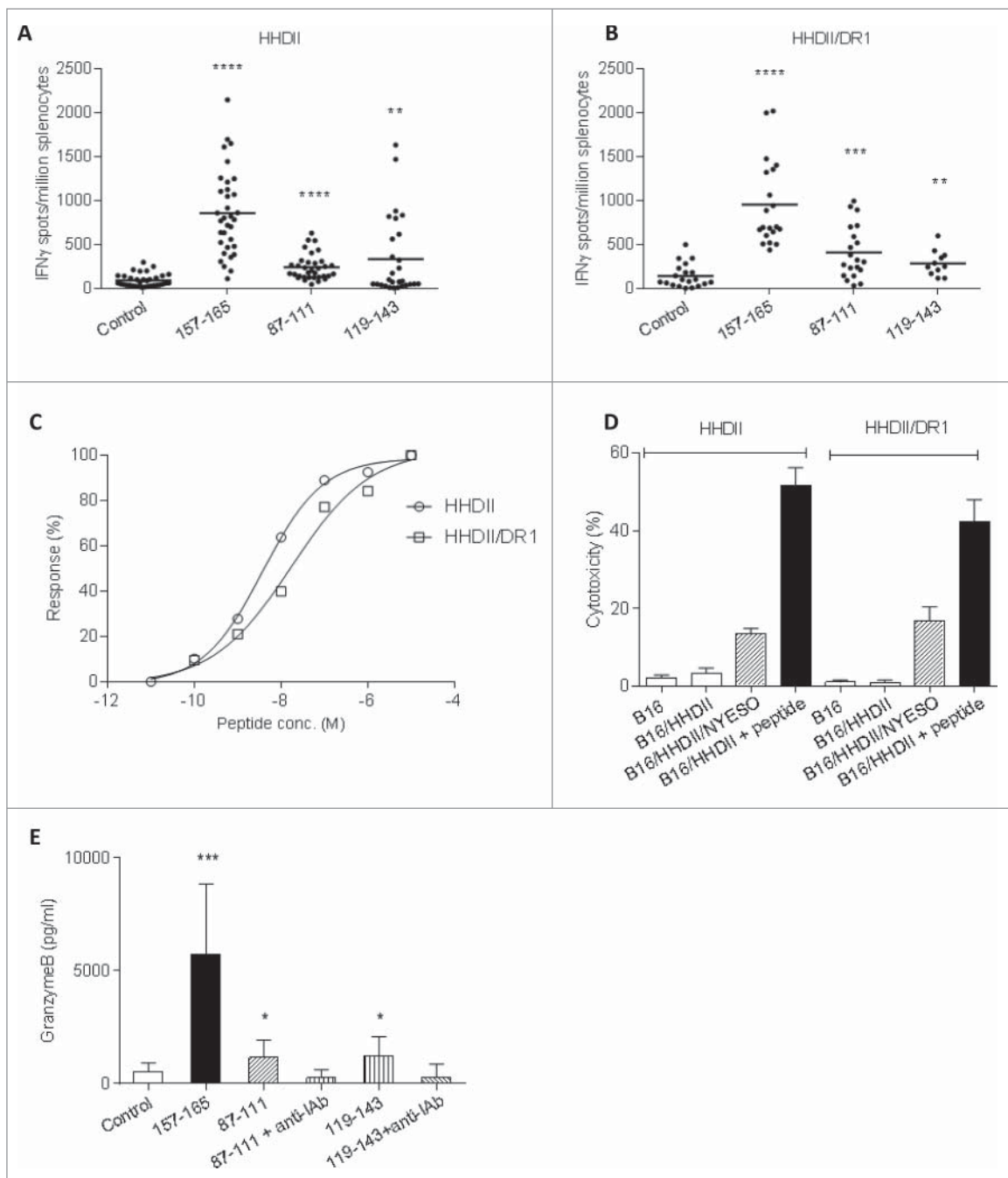


Figure 2. Epitope-specific responses generated in HHDII and HHDII/DR1 mice immunized with SCIB2. Splenocytes from SCIB2-immunized HHDII mice (A) and HHDII/DR1 mice (B) were analyzed by IFN γ Elispot to show the frequency of responses to NY-ESO-1 157–165, 87–111 and 119–143. Graph shows pooled data from >3 experiments in which $n = 3$. (C) Splenocytes from immunized HHDII and HHDII/DR1 mice were assayed for avidity to NY-ESO-1 157–165 peptide by measuring responses to increasing concentration of peptides in IFN γ Elispot assay. (D) After 6 d *in vitro* stimulation, cytotoxicity of NY-ESO-1 157–165-specific T cells on tumor lines were assessed by ^{51}Cr -release assay at a 50:1 effector: target ratio. (E) Granzyme B release from splenocytes of HHDII mice immunized with SCIB2. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Data show mean and SD and are representative of at least two experiments where $n \geq 3$.

SCIB2-induced T cell responses. Similar frequencies and avidities of CD8 $^{+}$ epitope-specific responses were observed with SCIB2 in the presence or absence of anti-CD25 Ab (Figs. 5A and B). The cytotoxicity and IFN γ release (Fig. 5C and Fig. S2) by SCIB2-induced T cells was also similar in the presence or absence of anti-CD25 Ab. This data implies that anti-CD25 Ab had little effect upon the induction of NY-ESO-1-specific CD8 $^{+}$ responses.

To determine if CTLA-4 or PD-1 blockade could enhance SCIB2-induced T cell responses, mice were immunized with SCIB2 in the presence or absence of anti-CTLA-4 or anti-PD-1

Ab. Anti-CTLA-4 and anti-PD-1 Ab did not significantly enhance the frequency (Figs. 6A and D) or avidity (Figs. 6B and E) of the CD8 $^{+}$ T cell responses. However, when splenocytes from SCIB2/anti-CTLA-4-immunized mice were incubated *in vitro* with NY-ESO-1 157 peptide, they showed significantly enhanced tumor killing of targets expressing NY-ESO-1 when compared to SCIB2 alone immunized mice (Fig. 6C). This suggests that anti-CTLA-4 Ab could enhance CTL cytotoxicity. In contrast, anti-PD-1 Ab did not enhance the CTL cytotoxicity killing over tumor targets (Fig. 6F).

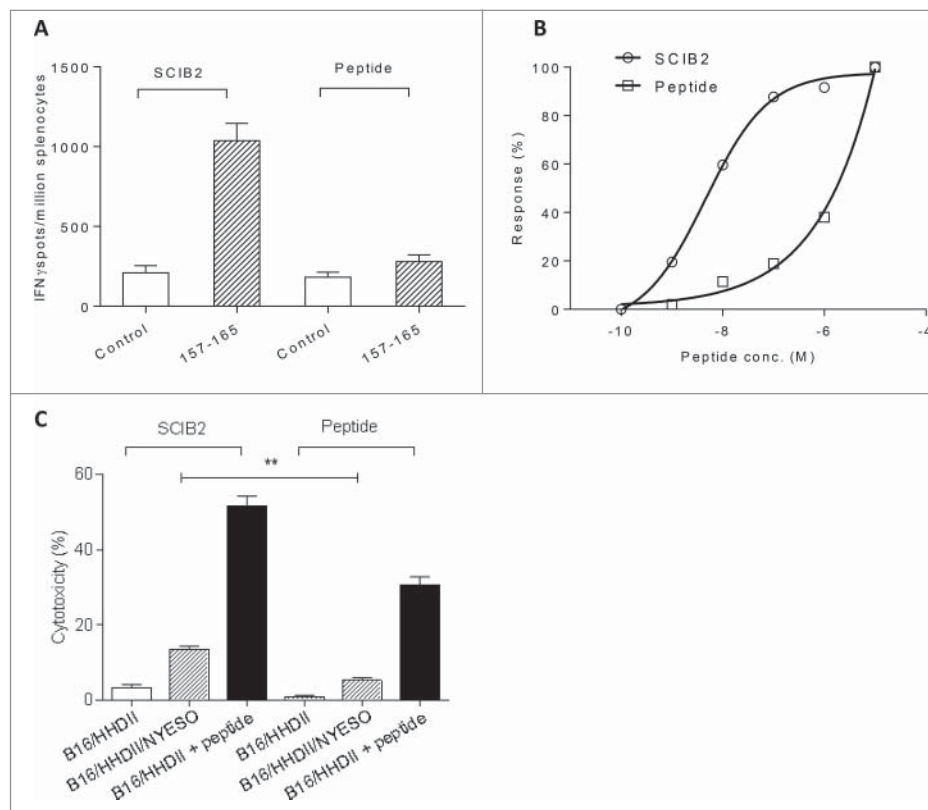


Figure 3. Immune responses and cytotoxicity induced in HHDII mice immunized with SCIB2 or NY-ESO-1 peptides. Comparison of (A) frequency and (B) normalized avidity responses to NY-ESO-1 157–165 peptide measured in IFN γ Elispot assay from mice immunized with either SCIB2 or peptide. (C) Cytotoxicity of splenocytes assessed by ^{51}Cr -release assay. Data are shown at effector to target ratio of 50:1. $**p < 0.01$. Data are presented as mean and SD and are representative of at least two experiments in which $n \geq 3$.

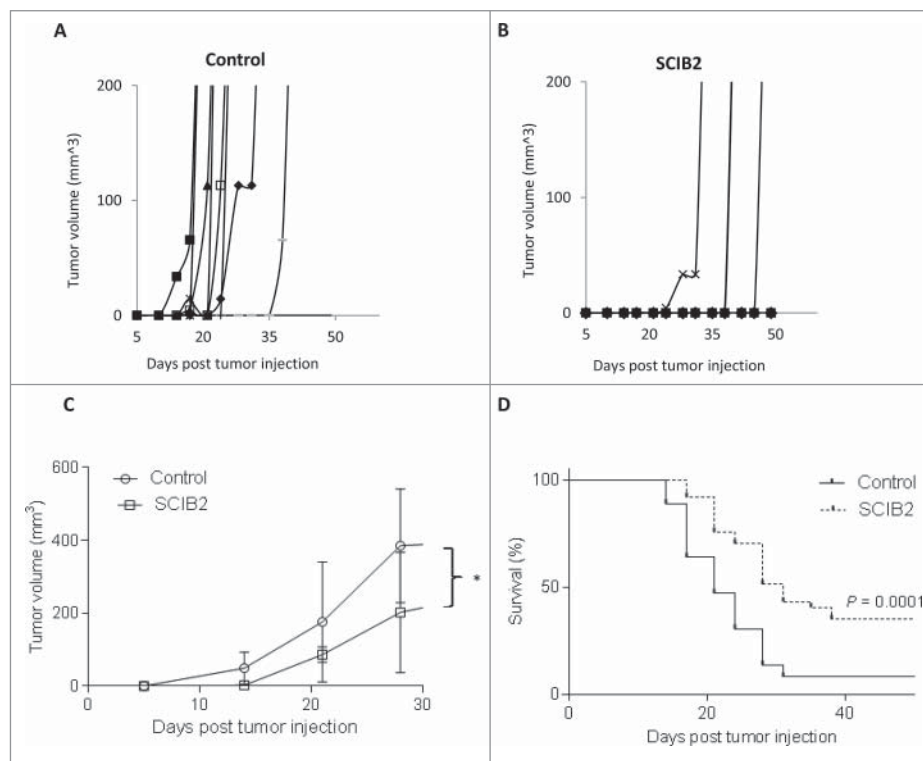


Figure 4. SCIB2 stimulates strong antitumor immunity. Individual (A and B) and average (C) tumor growth curves and percentage survival (D) of HHD II mice challenged with 2.5×10^4 B16/HHDII/NY-ESO-1 tumor cells at day 0 and immunized with SCIB2 at days 4, 11 and 18. $*p < 0.05$. Lack of survival was defined as tumor size $> 528 \text{ mm}^3$. (A) and (B) are representative of data from one study in which $n = 10$. (C) and (D) are representative of data from at least two independent studies in which $n = 10$ each study.

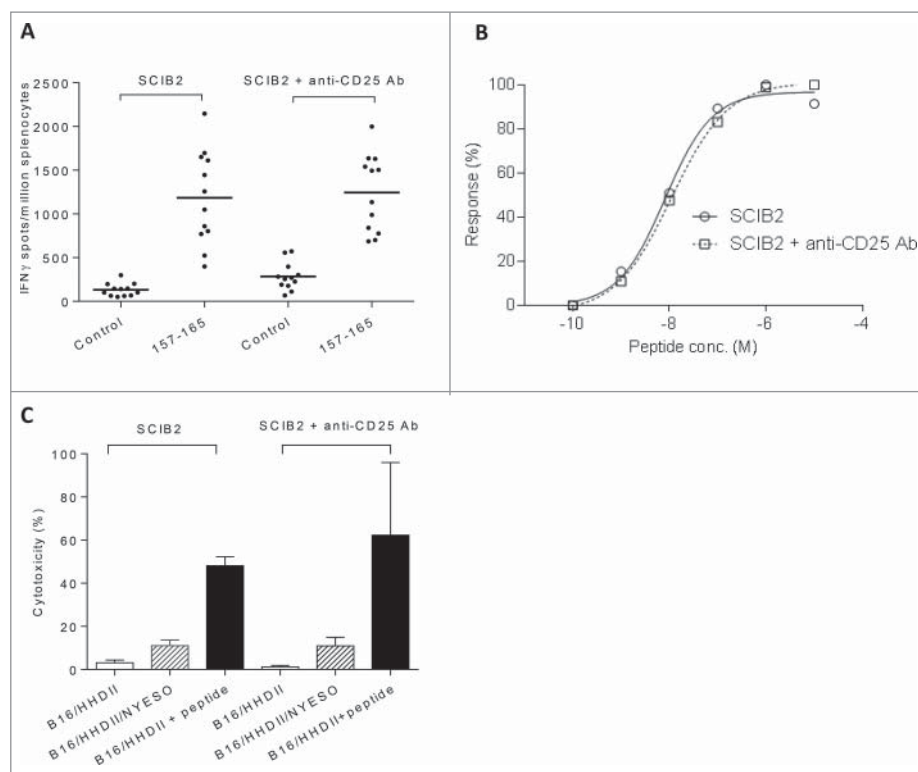


Figure 5. T cell responses induced by combining SCIB2 with Treg depletion. (A–C) Mice were immunized with SCIB2 in combination with anti-CD25 Ab. Splenocytes from immunized mice were assayed at day 20 for (A) frequency of immune responses against 10 μ g/mL of NY-ESO-1 157–165 peptide. Graph shows pooled data from >3 experiments in which $n = 3$. (B) Normalized avidity by titration of NY-ESO-1 157–165 peptide by IFN γ Elispot assay. (C) After 6 d *in vitro* stimulation with NY-ESO-1 157–165 peptide, CD8 $^{+}$ T cells were assessed by 51 Cr-release cytotoxicity assay. Data is shown at an effector to target ratio of 50:1. Data show mean and SD and are representative of at least two experiments, where $n \geq 3$.

Anti-CD25 Ab and checkpoint blockade enhance the antitumor effect of SCIB2

As addition of anti-CD25 Ab or checkpoint blockade did not enhance the SCIB2 vaccine-induced T cell responses, we assessed if these treatments could enhance tumor rejection. In order to ensure that the vaccine-induced T cells entering the tumor were protected from Treg depletion/blocking or checkpoint inhibitors, administration of anti-CD25 Ab was initiated at the same time as tumor challenge and the checkpoint blockade antibodies were given at the same time as vaccination.

The combination of anti-CD25 Ab and vaccination with SCIB2 was assessed in a therapeutic antitumor model (HHDII mice challenged with 2.5×10^4 B16/HHDI/YESO-1 tumor cells). Administration of anti-CD25 Ab at the same time as tumor challenge was not sufficient for tumor rejection ($p > 0.05$) (Fig. 7A). However, mice immunized with SCIB2 demonstrate enhanced survival compared to control ($p = 0.001$) (Fig. 7A). While the combination of anti-CD25 Ab and SCIB2 vaccination resulted in further inhibition of tumor growth compared to vaccination with SCIB2 ($p = 0.0484$) with 56% of mice showing long-term tumor-free survival (Fig. 7A).

To further assess the efficacy of combined vaccines, HHDII mice were given a higher tumor load of B16/HHDI/YESO-1 tumor cells (1.5×10^5) than in the previous experiment. SCIB2 alone and anti-CD25 Ab alone failed to show any therapeutic advantage compared to control (Fig. 7B) against these high tumor load. However, SCIB2 showed synergy with anti-CD25

Ab with 50% long-term survival (Fig. 7B). The combination group showed a significantly higher survival rate than the single therapies (SCIB2 vs. SCIB2 plus anti-CD25 Ab, $p = 0.03$; and anti-CD25 vs. SCIB2 plus anti-CD25, $p = 0.04$).

The combination of anti-CTLA-4 Ab and vaccination with SCIB2 was assessed in the therapeutic antitumor model. In the lower dose model (2.5×10^4 cells), anti-CTLA-4 Ab and SCIB2 alone significantly delayed tumor growth with long-term survival of more than 50% (Fig. 7C). This was further enhanced to 65% improvement of survival when these modalities were combined (Fig. 7C). Using a higher tumor cell dose (1×10^5 cells), both single therapy groups still showed a significant survival advantage over control (Fig. 7D). However, the combination therapy induced antitumor responses in 67% mice which is significantly enhanced when compared to single therapies (control vs. SCIB2, $p = 0.021$; SCIB2 vs. SCIB2+anti-CTLA-4 Ab, $p = 0.02$; anti-CTLA-4 Ab vs. SCIB2 + anti-CTLA-4 Ab, $p = 0.05$; control vs. SCIB2+anti-CTLA-4 Ab, $p < 0.0001$) (Fig. 7D).

The antitumor effect of combination of PD-1-blocking antibody and vaccination with SCIB2 was then assessed. Mice were challenged with (5×10^4) B16/HHDI/YESO-1 tumor cells; anti-PD-1 Ab and SCIB2 alone delayed tumor growth with long-term survival of 50% (Fig. 7E). This was further enhanced to 100% improvement of survival when these modalities were combined (control vs. SCIB2, $p = 0.037$; control vs. anti-PD-1 Ab, $p = 0.11$; SCIB2 vs. SCIB2+anti-PD-1 Ab, $p = 0.0177$; anti-PD-1 Ab vs. SCIB2 + anti-PD-1 Ab, $p = 0.0177$; control vs. SCIB2+anti-PD-1 Ab, $p = 0.0003$).

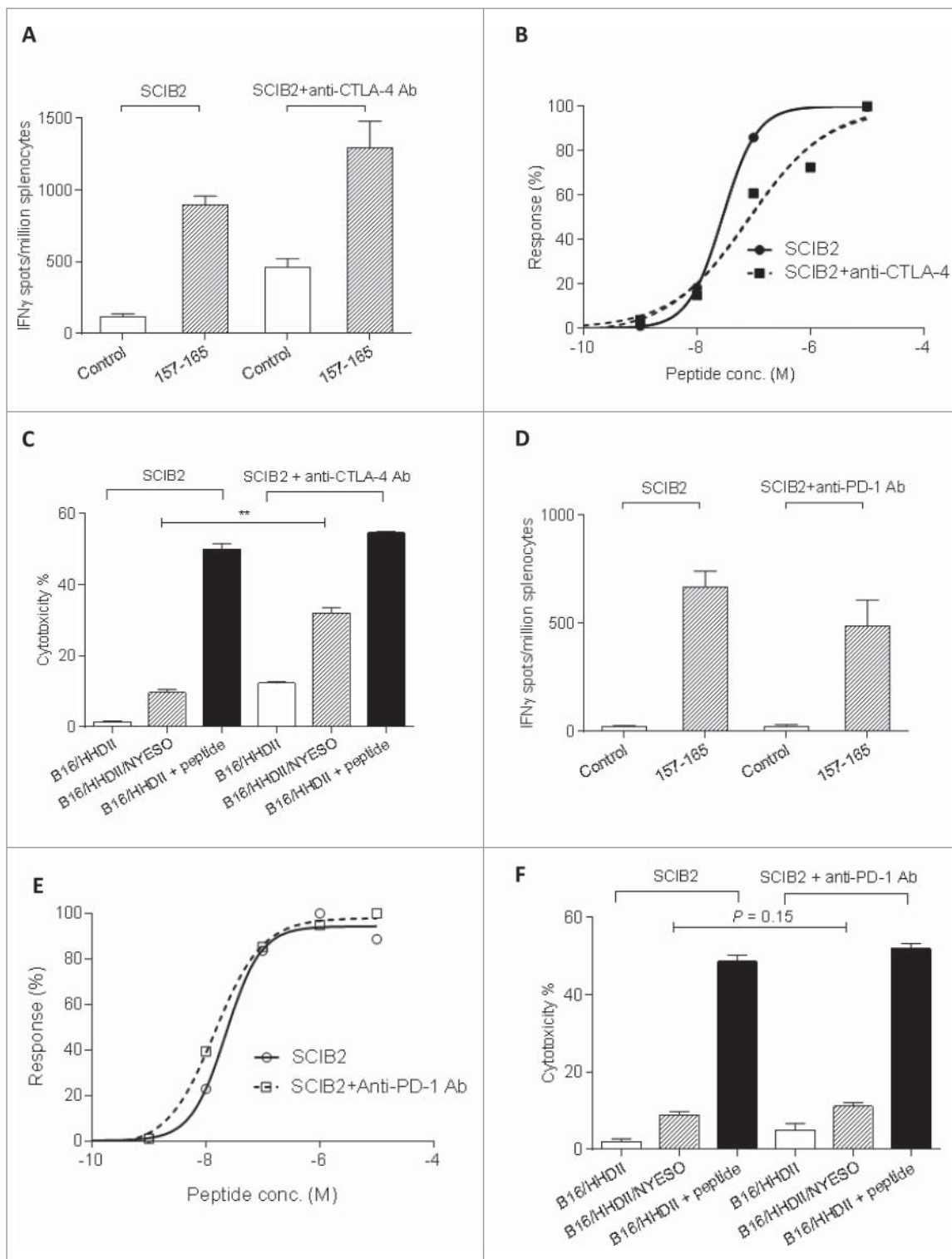


Figure 6. T cell responses induced by combining SCIB2 with checkpoint inhibitors. Mice were immunized with SCIB2 in combination with the anti-CTLA-4 Ab (A–C) or anti-PD-1 Ab (D–F). (A, D) Frequency of Immune responses against 10 μ g/ mL NY-ESO-1 157–165 peptide and (B, E) avidity by titration of NY-ESO-1 157–165 peptide were measured by IFN γ Elispot assay. (C, F) After 6 d *in vitro* stimulation with NY-ESO-1 157–165 peptide, CD8 $^{+}$ T cells were assessed for the ability to kill B16/HHDII/NY-ESO-I target cells by 51 Cr-release cytotoxicity assay. Data are shown at an effector to target ratio of 50:1. ** $p < 0.01$. Data are presented as mean and SD. Data are representative of at least two experiments in which $n \geq 3$.

Discussion

NY-ESO-1 is a cancer testis antigen that has a restricted normal expression but is expressed by a wide range of tumors.^{7–9} It is very immunogenic and many patients have endogenous

responses to this antigen^{11,12,24,52} as we confirmed in our *in vitro* studies from melanoma patients. We showed T cell response to one or more of 16 T cell epitopes in 82–90% of patients. Despite the presence of spontaneous T cell responses, the majority of patients still die of their disease. One of the key

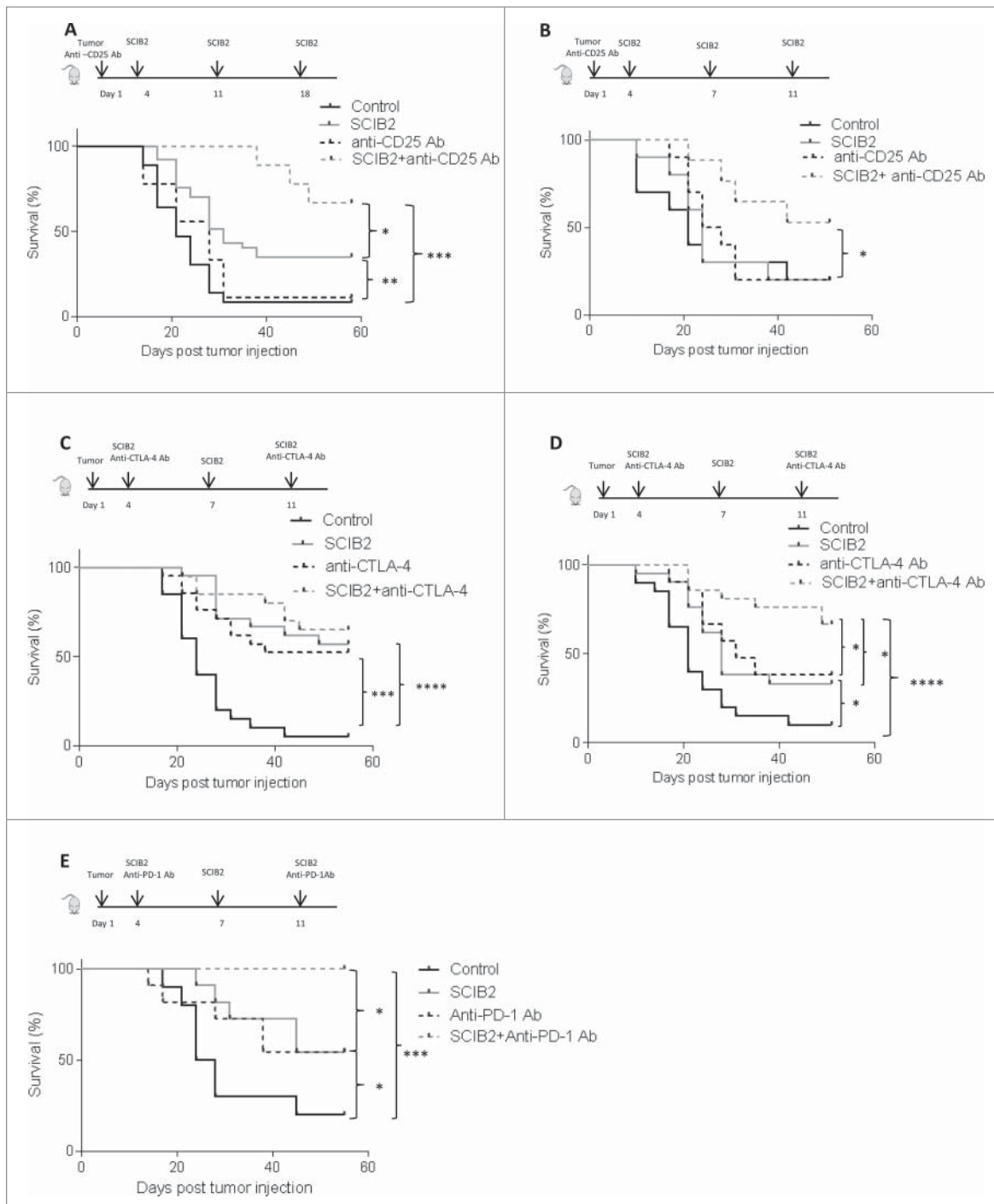


Figure 7. Antitumor effect of combination of SCIB2 and Treg depletion and SCIB2 with CTLA-4 or PD-1 blockade. HHDII mice were implanted with B16/HHDII/NY-ESO-1 cells and treated with anti-CD25 Ab, SCIB2 or both. (A) Percentage survival of mice challenged with low-dose (2.5×10^4) tumor and immunized with SCIB2 and anti-CD25 Ab alone or in combination. Con vs. SCIB2 (** $p = 0.001$); SCIB2 vs. SCIB2+anti-CD25Ab depletion (* $p = 0.0484$); Con vs. SCIB2+anti-CD25 Ab (** $p = 0.0006$). (B) Percentage survival rate of mice challenged with high-dose (1.5×10^5) tumor. Con vs. SCIB2 ($p > 0.05$); Con vs. anti-CD25 Ab ($p > 0.05$); SCIB2 vs. SCIB2+anti-CD25 Ab depletion (* $p = 0.03$); anti-CD25 Ab vs. SCIB2+anti-CD25Ab (* $p = 0.04$); (C) Survival of mice challenged with 2.5×10^4 tumor cells and immunized with SCIB2 and anti-CTLA-4 Ab alone or in combination. Con vs. anti-CTLA-4 Ab (** $p = 0.0003$); Con vs. SCIB2 (**** $p < 0.0001$); anti-CTLA-4 Ab vs. anti-CTLA-4 Ab plus SCIB2 ($p > 0.05$); SCIB2 vs. SCIB2 plus anti-CTLA-4 Ab ($p > 0.05$); (D) shows survival of mice challenged with 1×10^5 tumor cells and immunized with SCIB2 and anti-CTLA-4 Ab alone or in combination. Con vs. SCIB2 (* $p = 0.021$); SCIB2 vs. SCIB2+anti-CTLA-4 Ab (* $p = 0.02$); anti-CTLA-4 Ab vs. SCIB2 + anti-CTLA-4 Ab (* $p = 0.05$); Con vs. SCIB2+anti-CTLA-4 Ab (**** $p < 0.0001$); (E) Survival of mice challenged with 5×10^4 tumor cells and immunized with SCIB2 and anti-PD-1 Ab alone or in combination. Con vs. SCIB2 (* $p = 0.037$); Con vs. anti-PD-1 Ab ($p = 0.111$); Con vs. SCIB2+anti-PD-1 Ab (**** $p = 0.0003$); SCIB2 vs. SCIB2+anti-PD-1 Ab (* $p = 0.0177$); anti-PD-1 Ab vs. SCIB2+anti-PD-1 Ab (* $p = 0.0177$). Lack of survival was defined as tumor size $> 528 \text{ mm}^3$. Each curve represents at least 10 mice per group.

factors may be failure to generate high-avidity T cells that can lyse tumor cells. We have previously demonstrated the superiority of the vaccine approach of encoding antigenic epitopes within a human antibody IgG1 framework for the induction of

high-avidity CD8⁺ and CD4⁺ responses.^{18,19} SCIB2 was therefore designed to encode these 16 NY-ESO-1 epitopes, which are nested within four regions of NY-ESO-1, in the CDR regions of the human IgG1 Ab ImmunoBody® vector.

SCIB2 was tested in HHDII (HLA-A*0201) transgenic mice models. It not only elicited a higher frequency and avidity response to NY-ESO-1 157 than peptide vaccination but also generated CD8⁺ responses with the ability to recognize naturally processed and presented NY-ESO-1 antigen on the surface of tumor cells. This is consistent with our previous findings that the frequency and avidity of responses from SCIB1 was significantly higher than that from peptide immunization.¹⁹ In addition, SCIB2 induced high-frequency CD4⁺ responses in HHDII and HHDII/DR1 transgenic mice confirming I-Ab and demonstrating HLA-DR1 restriction.^{10,12,25-27} Furthermore, the induced CD4⁺ cells released granzyme B that could be completely blocked by MHC class II Ab suggesting that they were cytotoxic CD4⁺ responses. Recent studies demonstrate CD4⁺ T cells can possess cytotoxicity abilities and are able to lyse virus-infected and tumor cells.^{28,29} NY-ESO-1 87–111-specific CD4⁺ CTL lines from cancer patients have also been shown to recognize autologous APC loaded with protein or transfected with NY-ESO-1 cDNA.¹² MHC II expression can be induced in majority of cells, including melanoma by IFN γ ; it can also be constitutively expressed in some melanomas.³⁰⁻³² The effect of both CD8⁺ and CD4⁺ responses on tumor therapy was measured using the HHDII transgenic mouse model. As NY-ESO-1 does not have a murine homolog, the human NY-ESO-1 was cloned into the B16/HHDII cell line. As a foreign antigen, it was anticipated that it may induce an antitumor response in the absence of vaccination. However, NY-ESO-1-expressing tumors still grew rapidly in the majority of mice. In contrast, the strong cytotoxic high-avidity NY-ESO-1-specific CD8⁺ and CD4⁺ T cell responses induced by SCIB2 led to long-term survival in 35% of mice. In this study, we did not investigate if the antitumor effect induced by SCIB2 was CD4⁺ and/or CD8⁺ T cells mediated, this will be examined in a future study. We have shown both CD8⁺ and CD4⁺ responses to NY-ESO-1 epitopes encoded within SCIB2 DNA constructs could be generated in patients which gives a good indication that SCIB2-stimulated responses in humans may involve both CD8⁺ and CD4⁺ T cells.

Tumors are well known to promote an anti-inflammatory suppressive environment and recent data has demonstrated that high-avidity CD8⁺ T cells are tolerized within the tumor environment rendering them non-functional.³³ We report that SCIB2-mediated antitumor responses were further enhanced in combination with Treg depletion/blocking. Anti-CD25 Ab has been used in many studies to deplete/block Tregs in murine models and reports suggest an antitumor role as a monotherapy or combined with vaccines.^{34,35} However, its antitumor efficacy as a monotherapy was limited in aggressive tumor models, including B16.⁶⁴ Daclizumab, a human anti-CD25 Ab has been shown to reduce the presence of Tregs in peripheral blood; however, its antitumor therapeutic effect has yet to be determined as it can also eliminate activated T cells.³⁶ A recent clinical trial demonstrates the synergistic effect of cyclophosphamide with multiple peptide vaccines in renal cancer patients with prolonged survival.³⁷ This effect was not observed in the absence of immune responses which suggest the importance of both immune response and immunomodulatory mechanisms.

A vaccination that generates high-avidity T cells could be used in cancer treatments along with checkpoint inhibitors to

remove the brakes and unleash the full potential of the T cell response. Indeed, we have shown here that in a preclinical model, the combination of CTLA-4 with SCIB2 resulted in an enhanced antitumor response. This is in contrast to other studies showing that when it is given as monotherapy, anti-CTLA-4 Ab failed to reject tumor in several models (B16, SM1 mammary carcinoma, EL4 lymphoma, M109 lung cancer).³⁸ CTLA-4 binds to CD80/86 with higher affinity than CD28 and inhibits the activating signals. Using our immunization regime of co-vaccinating with the initial and third dose of vaccine, we showed no increase in the frequency or the avidity of the SCIB2-induced immune response but did see good additive antitumor responses. This is in line with other studies showing that T cell responses can be suppressed within the tumor environment and anti-CTLA-4 Ab can reactivate T cells at the tumor site.³⁹

In our preclinical model, the combination of anti-PD-1 with SCIB2 resulted in an enhanced antitumor response. Studies in the B16 tumor model in C57Bl/6 mice have failed to show an effect with PD-1 blockade alone.⁴⁰ This may be a reflection of its low immunogenicity and/or the fact it does not normally express PD-L1 *in vivo*. Previous studies indicate that the PD-L1 level within the tumor environment can be elevated by infiltrating activated T cells secreting IFN γ .^{41,42} PD-L1 expression within tumor environment can be related to poor prognosis in various cancers.⁴³⁻⁴⁵ High levels of PD-L1 expression in the tumor microenvironment have also been correlated with a better response rate to PD-1/PD-L1 blockade in cancer patients.⁴⁶ However, some patients whose tumors do not express PD-L1 also respond to PD-1/PD-L1 blockade. PD-L1 can also be expressed on infiltrating monocytes and endothelial cells and the role these cells play in checkpoint inhibition remains unclear. It is suggested that induction of PD-L1 in the melanoma tumor microenvironment may suppress T cells mediated antitumor immune responses by engaging PD-1 on the tumor-infiltrating T cells⁴¹ and may also promote iTreg induction, proliferation and immune suppressive function.⁴⁸ Recent studies showed, in addition to blocking negative pathway in immune cells, the checkpoint inhibitors (anti CTLA-4 and anti PD-1 Abs) also eliminates Treg.^{40,49,50} Within the tumor environment, depletion of Tregs by anti-CTLA-4 is dependent on the presence of Fc γ receptor-expressing receptor macrophage.⁵⁰ This is consistent with our results in which anti-CTLA-4 and anti-PD-1 Abs failed to increase the immune response to SCIB2 but did enhance its antitumor response with 100% of mice showing long-term survival with the SCIB2/anti-PD-1 combination.

In conclusion, this study shows that targeting NY-ESO-1 epitopes to antigen-presenting cells results in high-avidity and high-frequency T cell immune responses, with potent antitumor responses, which were further enhanced by checkpoint blockade. The 100% tumor survival with the combination of SCIB2 and PD-1 blockade is especially promising as PD-1 blockade is known to have lower associated toxicity when compared to CTLA-4 blockade. SCIB2 will now be rapidly translated into the clinic where it is anticipated that patients with low tumor burden may benefit from SCIB2 alone but patients with more bulky disease may benefit from combinations of SCIB2 with checkpoint blockade.

Materials and methods

Vaccine and expression plasmids

In brief, to generate SCIB2 oligonucleotides encoding the HLA-A24 epitope 158–166 (LLMWITQCF) and HLA-A2-restricted epitope 157–165 (SLLMWITQC) were incorporated into the CDRH1 and CDRH2 of the vector pDCOrig, respectively, as previously described.¹⁸ The NY-ESO-1 amino acid sequence 83–111 (PESRLLEFYLPMPFATPMEALARRSLAQ) was cloned into the CDRH3 site. NY-ESO-1 amino acid sequence 119–143 (PGVLLKEFTVSGNILTIRLTAAADHR) was inserted into the CDRHL1 site within the light chain.

To construct the mammalian double expression plasmid that encodes murine TAP2 and NY-ESO-1, NY-ESO-1 was amplified from the IMAGE clones 40146393 obtained from Genservice with forward and reverse primers that incorporated a BamHI/XhoI site, respectively. On sequence confirmation, full-length NY-ESO-1 was ligated into the BamHI/XhoI multiple cloning site of the double expression vector in replacement of the light chain. Murine TAP2 (after site-directed mutagenesis of the image clone 6530488 to remove a HindIII site from encoding sequence and insertion before the start codon) was cloned into the expression vector pOrigHIB using HindIII/EcoRV, then transferred in replacement of the heavy chain using HindIII/AvrII into the double expression vector alongside full length NY-ESO-1.

To generate the HHD plasmid, cDNA was synthesized from total RNA isolated from EL4-HHD cells. This was used as a template to amplify HHD using the forward and reverse primers and subcloned into pCR2.1. The HHD chain, comprising of a human HLA-A2 leader sequence, the human β 2-microglobulin (β 2M) molecule covalently linked via a glycine serine linker to the α 1 and 2 domains of human HLA-0201 MHC class I molecule and the α 3, transmembrane and cytoplasmic domains of the murine H-2Db class I molecule, was then inserted into the EcoRV/HindIII sites of the mammalian expression vector pCDNA3.1 obtained from Invitrogen.

In order to knockdown expression of murine β 2M and murine MHC class II in the cell line B16F10, RNA interference was utilized. Complimentary oligonucleotides that target sequence 266 of murine β 2M and 159 of murine MHC class II were annealed and inserted separately into pCDNA6.2 GW miR (Invitrogen). The pre-miRNA expression cassette containing miRNA 266 was excised using BamHI/XhoI and ligated into the XhoI/BglII site of pCDNA6.2 GW miR 159 in order to chain the two miRNAs and express them in one primary transcript within the same vector.

Cell lines

B16F10 cell line (ATCC) was maintained in RPMI (Cambrex) with 10% FBS (Sigma). B16F10 cells were transfected successively using Lipofectamine transfection reagent (Invitrogen) with expression vectors encoding full-length NY-ESO-1 and Murine TAP2, HHDII and a siRNA to knockdown expression of murine MHC class II and murine β 2M. Transfected cells were selected by growth in the presence of Zeocin (300 μ g/ mL), G418 (500 μ g/ mL) and Blastidicin (4 μ g/ mL), respectively. Lines

were cloned by limiting dilution and expression was confirmed by flow cytometry.

Mice and immunizations

Animal work was carried out under a Home Office approved project license at Nottingham Trent University. HLA-A2/DR1 (HHDII/DR1) or HLA-A2 (HHDII) transgenic mice on a C57Bl/6 background (Pasteur Institute) were used between 6 and 16 weeks of age. HHDII mice are murine class I deficient and express the human HLA-A2 allele. HHDII/DR1 mice are deficient in both murine MHC class I and II and replaced with the human HLA-A2 and DR1 alleles. Synthetic peptide SLLMWITQC (NY-ESO-1 157–165) was emulsified with IFA/CFA combination (Invivogen). Peptide (25 μ g/immunization) was injected via the subcutaneous route at the base of the tail. DNA was coated onto 1.0 μ m gold particles (Bio-Rad) using the manufacturer's instructions and administered intradermally by the Helios Gene Gun (Bio-Rad). Each mouse received 1 μ g DNA/immunization into the shaved abdomen. Depletion of Tregs was performed by intraperitoneal injection of 400 μ g anti-CD25 Ab (PC61) (BioXcell) 4 d prior to the first immunization. Mice were immunized at day 1, 8 and 15 and spleens removed at day 20 for analysis, unless otherwise stated. The half-life of the hamster anti-CTLA-4 (9H10) Ab *in vivo* has been shown to be 3–4 d.⁵¹ The rat anti PD-1 (RMP1-14) Ab has a slightly longer half-life and is usually administered every 4–7 d. For consistency, we gave both checkpoint blockade antibodies weekly with the first and last SCIB2 administration. Anti-CTLA-4 Ab (9H10) or anti-PD-1 Ab (RMP1-14) (BioXcell) was injected at 200 or 250 μ g intraperitoneally at day 1 and 15.

Proliferation assay

PBMC from melanoma patients were isolated by Ficol-Hypaque (Sigma) gradient centrifugation. In brief, PBMC (1.5×10^6 cell/well) were stimulated with peptide (10 μ g/ mL) for 10 d at 37°C. The cultured PBMC were then incubated with ³H-thymidine (0.0185 MBq/well) for 8 h at 37°C. The cultures were harvested onto unfilter plates and incorporation of ³H-thymidine was determined by β -scintillation counting. The results were assessed by calculating the proliferation index (PI) as the ratio of the mean of counts per minutes (cpm) of epitope-stimulated to the mean of unstimulated cultures. The proliferative assay was considered positive when PI > 2.5.

Ex vivo Elispot assay

Elispot assays were performed using murine IFN γ capture and detection reagents according to the manufacturer's instructions (Mabtech AB). Detailed method was described previously.¹⁹ Synthetic peptides SLLMWITQC (NY-ESO-1 157–165), LLEFYLPMPFATPMEALARRSLAQ (NY-ESO-1 87–111) and PGVLLKEFTVSGNILTIRLTAAADHR (NY-ESO-1 119–143) (at a variety of concentrations) were used in these assays. Anti-mouse MHC-II IAb blocking Ab (M5/114.15.2) was purchased from ebioscience. Anti HLA-DR blocking Ab (L243) was obtained from Biolegend.

Re-stimulation in vitro

Splenocytes (5×10^6 /mL) were co-cultured at 37°C with irradiated, peptide-pulsed LPS blast. LPS blasts were obtained by activating splenocytes (1.5×10^6 /mL) with 25 µg/mL LPS (Sigma) and 7 µg/mL dextran sulfate (Pharmacia) for 48 h at 37°C. Before use, 2×10^7 LPS blasts were labeled with 10 µg/mL synthetic peptide for 1 h. Anti CTLA-4 Ab and PD-1 Ab were used at 40 µg/mL. After six days, cultures were assayed for cytotoxic activity in a ^{51}Cr -release assay.

CTL assay

Target cells were labeled for 90 min with 1.85 MBq sodium (^{51}Cr) chromate (Amersham) in the presence or absence of 1 µg/mL (NY-ESO-1 157) peptides. Target cells (5×10^3 /well) were then incubated with different numbers of effector cells in a final volume of 200 µL. After 4 h incubation at 37°C, 50 µL of supernatants were transferred to a Lumaplate (Perkin Elmer). Plates were then read on a Topcount Microplate Scintillation Counter (Packard). Percentage-specific lysis was calculated using the following formula: specific lysis = $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$.

Granzyme B ELISA

Supernatant from *ex vivo* IFN γ Elispot assays was analyzed using a granzyme B Elisa kit according to manufacturer's instruction (R&D system).

Tumor studies

In the therapeutic model, HLA-A2 transgenic (HHDII) mice were challenged by subcutaneous injection with 2.5×10^4 B16/HHDII/NY-ESO-1 cells at day 1. Anti-CD25 Ab (400 µg/mouse) was injected intraperitoneally concurrent with tumor cell implant on day 1. The vaccination groups were immunized with DNA bullets intradermally using the Helios Gene Gun for three consecutive weeks at day 4, 11 and 18. For a more aggressive tumor model, 1.5×10^5 B16/HHDII/NY-ESO-1 cells was injected on day 1. For checkpoint blockade tumor model, the vaccine groups were immunized with DNA bullets at day 4, 7 and 11. Anti-CTLA-4 Ab (200 µg/mouse; BioXcell) or anti-PD-1 (250 µg/mouse, BioXcell) was injected intraperitoneally on day 4 and 11. The end point for tumor therapy studies was when tumor exceeded 528 mm³.

Statistical analysis

Comparative analysis of the Elispot, Elisa, CTL killing assay and tumor size results was performed by applying the student's *t* test with values of *p* calculated accordingly. Paired Wilcoxon ranking test was used to compare peptide with media Elispot responses from the same mice. Comparison of avidity curves and tumor growth was performed by applying the two-way ANOVA and survival studies were assessed by Log Rank test using the Graphpad Prism 5.0 software (GraphPad Software, Inc.). *p* < 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

The authors wish to disclose that Lindy G. Durrant is a director of Scancell Ltd and W. X., R. L. M., V. A. B., P. S., and B. G. are employees of Scancell Ltd.

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