Article type : Original Article

ImmunoBody®-HAGE derived vaccine induces immunity to HAGE and delays the growth and metastasis of HAGE-expressing tumours *in vivo*

Divya Nagarajan[1,3], Joshua Pearson [2,3], Victoria Brentville [4], Rachael Metheringham [4], A Graham Pockley[1,2], Lindy Durrant[4], Stephanie E McArdle[1,2*].

[1] Department of Immunology, Genetics and Pathology, Uppsala University, Sweden

[2] John van Geest Cancer Research Centre, School of Science and Technology, Nottingham Trent University, Nottingham, UK

[3] Centre for Health, Ageing and Understanding Disease, School of Science and Technology, Nottingham Trent University, Nottingham, UK

[4] Scancell Ltd, Biodiscovery Institute, University of Nottingham, University Park, NG7 2RD, UK

Running head: ImmunoBody®-HAGE vaccine to prevent triple-negative breast cancer relapses

(ALLOW A LONG RUNNING HEAD)

***Correspondence**: stephanie.mcardle@ntu.ac.uk, Tel.: +44 115-848-3136

Keywords: Triple-negative Breast Cancer (TNBC), HAGE, vaccine, metastasis, treatment

ABSTRACT

The management of patients with triple-negative breast cancer (TNBC) continues to pose a significant clinical challenge. Less than 30% of women with metastatic TNBC survive 5 years, despite adjuvant chemotherapy and the initial higher rates of clinical response that can be achieved with neoadjuvant chemotherapy. ImmunoBody® is a plasmid DNA designed to encode a human antibody molecule with complementary determining regions (CDRs) engineered to express

cytotoxic and helper T cell epitopes derived from the cancer antigen of interest. HAGE is a Cancer Testis Antigen, which is expressed in TNBC. Herein, we have identified a 30-amino-acid-long HAGE-derived sequence containing HLA-A2 and HLA-DR1 restricted epitopes and demonstrated that the use of this sequence as peptide (with CpG/IFA) or incorporated into an ImmunoBody® vaccine can generate specific IFNγ secreting splenocytes in HHDII/DR1 mice. T-cell responses elicited by the ImmunoBody®-HAGE vaccine were superior to peptide immunisation. Moreover, splenocytes from ImmunoBody®-HAGE vaccinated mice stimulated *in vitro* could recognise $HAGE^+$ tumour cells and the human TNBC cell line MDA-MB-231. More importantly, the growth of implanted B16/HHDII/DR1/$HAGE^+$ cells was significantly delayed by the ImmunoBody®-HAGE vaccine in both prophylactic and experimental metastasis settings. Overall, we demonstrate the potential of HAGE-derived vaccines for treating HAGE-expressing cancers and that such vaccines could be considered as therapeutic options for patients with $HAGE^+$ TNBC after conventional treatment to prevent disease recurrence.

INTRODUCTION

Breast cancer accounts for 28.2% of all cancer cases in European females and 16.2% of deaths in European women[1]. Triple-negative breast cancers (TNBC) constitute 10–20% of all breast cancers, and more frequently affect younger patients and women of African heritage. TNBCs are generally larger in size, are of higher grade, have lymph node involvement at diagnosis, and are biologically more aggressive. The management of TNBC remains a significant clinical challenge due to the lack of targeted therapies, with patients having a poorer prognosis and relapsing more frequently than patients with hormone receptor-positive subtypes of the disease. Less than 30% of women with metastatic TNBC survive 5 years, and almost all die from their disease despite adjuvant chemotherapy and the initial higher rates of clinical response that can be achieved with neoadjuvant chemotherapy[2]. The majority of TNBCs metastasise to the lungs, liver, bone and the brain, with brain metastases being particularly difficult to treat and associated with the poorest survival[3]. More advanced, effective treatments for TNBC are therefore urgently needed.

The aim of cancer vaccine-based immunotherapy is to induce protective, robust and sustained anti-tumour immunity against relevant target antigens. Success is critically dependent on identifying appropriate tumour antigens based on their specificity and frequency of expression in cancer tissues.

Cancer Testis Antigens are almost exclusively expressed by the testes and/or placenta in healthy adult tissues, with their expression in other settings being primarily restricted to malignant tumours. These tumour-restricted expression and immunogenic features make CTAs ideal candidates as targets for immunotherapy. The helicase antigen HAGE (DEAD-box protein DDX43) has previously been shown to be expressed in 14.8% of early primary TNBC tumours and 43% of locally advanced primary TNBC[4]. The role of HAGE as a potential prognostic marker for patients with breast cancer and a predictor of response to adjuvant chemotherapy has also been demonstrated[4]. Herein, we have investigated HAGE as a potential therapeutic target for CTA-targeted immunotherapy for TNBC expressing HAGE.

Vaccination using a peptide sequence within a native protein which is predicted to be immunogenic can maximise the probability of inducing both helper and cytotoxic T cell responses. Among several algorithms, the SYFPEITHI (http://www.syfpeithi.de) database enables the identification of immunogenic peptides within a protein sequence based on the presence and position of anchor residues that influence their binding affinities to different HLA alleles.

The ease of synthesis and generation of large quantities of peptide or DNA vaccines and their relatively low cost have made these attractive formats for anti-cancer vaccines. Moreover, the use of long peptides incorporating epitopes for CD8 rather than using only CD8-specific epitopes, or the whole protein has been shown to be superior at inducing $CD8^+$ T-cells [5, 6]. The use of long peptides also enables the possible presentation of $CD4^+$ specific T-cell epitopes. The use of adjuvants such as incomplete Freund's adjuvant (IFA) and CpG-ODN (TLR9 agonist) promotes the recognition of these peptides by professional antigen-presenting cells (APCs), thereby increasing antigen cross-presentation and promoting Th1-type immunity[7]. However, although many of these peptide/adjuvant vaccines have been reported to generate $CD8^+$ T cell responses, significant clinical efficacy has yet to be observed [8, 9].

Delivery of antigen or antigenic fragments using viral or plasmid DNA vectors has received much attention in recent years. The ImmunoBody® vaccine is a plasmid DNA designed to encode a human antibody molecule with complementary determining regions (CDRs) engineered to express cytotoxic and helper T cell epitopes derived from the antigen of interest [10, 11]. In the case of the study presented herein, these epitopes were derived from the HAGE sequence. ImmunoBody® -DNA vaccines have been shown to maximise T cell activation and the avidity of reactive cells by two distinct mechanisms: 'direct' and 'cross-presentation'. Direct presentation is achieved by the direct transfection of APCs and cross-presentation by secretion of antibody which efficiently stimulates helper and cytotoxic T lymphocyte (CTL) responses by effective targeting of dendritic cells (DCs) via the high-affinity Fc receptors. The first developed ImmunoBody®, called SCIB1, is a DNA plasmid that encodes a human antibody molecule engineered with T cell epitopes (both helper and cytotoxic) derived from melanoma antigens TRP-2 and gp-100 within the CDRs. The SCIB1 ImmunoBody® is currently being evaluated as a therapeutic for melanoma in Phase I/II clinical trials and has been shown to significantly prolong survival rates, particularly in patients following the resection of disease [12, 13].

The release of IFN-γ by vaccine-induced T-cells in response to tumour cells can induce or increase the expression of Programmed Death Ligand 1 (PD-L1) by the tumour cells which, in turn, will down-regulate the effector functions of activated T-cells expressing PD-1 via PD-L1/PD1 binding. We and others have previously shown that TNBCs exhibit a high level of tumour infiltrating lymphocytes (TILs) and a higher expression of the immunoregulatory checkpoint pathway ligand PD-L1 than other breast cancer subtypes [4, 14, 15], and that the presence of TILs is associated with a favourable outcome. Antibody-mediated inhibition of PD-L1/PD-1 interactions (so-called 'checkpoint inhibition') combined with cytotoxic chemotherapies that induce immunogenic tumour cell death have been shown to induce an objective response in

39.4% of patients with metastatic TNBC [16]. The checkpoint receptor PD-1 (Programmed Cell Death Protein 1) is primarily expressed by activated T cells, the effector functions of which are down-regulated upon PD-L1 engagement. Consequently, the efficacy of PD-1 and/or PD-L1 checkpoint inhibitors relies on the expression of PD-1 on immune effector cells and PD-L1 on tumour cells. However, if the patient's immune system has not been sufficiently stimulated, their T-cells will not express PD-1 and their tumour is also unlikely to have been induced to express PD-L1, except for tumours expressing a mutated PTEN which has been shown to induce PD-L1 expression [17]. Antigen/specific vaccination is therefore an approach that is likely to activate T-cells and initiate the development of protective anti-tumour immunity.

The main aim of this study was to identify regions within the HAGE protein that harbour immunogenic epitopes having the potential to trigger $CD4^+$ and $CD8^+$ T cell responses that can kill tumour cells expressing HAGE *in vitro* and tumours expressing HAGE *in vivo*. Herein, we have identified a 30 amino acid long immunogenic HAGE-derived sequence. Immune responses induced by a HAGE-30mer peptide-based vaccine and a HAGE ImmunoBody®-DNA vaccine (ImmunoBody®-HAGE) were then compared. ImmunoBody® vaccines are designed to generate potent high avidity T cell responses capable of a broad anti-tumour effect. We have shown that immunising animals with ImmunoBody®-HAGE (287-316) induced higher frequencies of HAGE peptide-specific T cells in the spleen than the peptide-adjuvant vaccine. Splenocytes from ImmunoBody®-HAGE vaccinated mice stimulated *in vitro* could recognise $HAGE^+$ tumour cells (B16/HHDII/$DR1^{HAGE+}$) and the human TNBC cell line MDA-MB-231. More importantly, the growth of implanted B16/HHDII/$DR1^{HAGE+}$ cells was significantly delayed by the ImmunoBody®-HAGE (287-316) vaccine in both prophylactic and experimental metastasis settings. Overall, we demonstrate the potential of HAGE-derived vaccines for treating HAGE-expressing cancers and that such vaccines could be considered as therapeutic options for patients with TNBC expressing HAGE after conventional treatment to prevent disease recurrence.

RESULTS

Immunogenicity of the HAGE-derived 30mer peptide

The SYFPEITHI database searching tool identified a 30mer HAGE-derived sequence encompassing several peptides that were predicted to bind to different HLA haplotypes including HLA-A2, A1, A3, DR1 and DR4 with high affinity. The binding scores for these sequences are shown in Table 1.

In order to determine the immunogenicity of the HAGE-derived 30mer sequence, as well as to compare the efficacy of two most commonly used adjuvants - incomplete Freund's adjuvant (IFA) [18-21] and CpG ODN 1826 [22-24], these were tested individually and together with the vaccine. HHDII/DR1 mice were immunised with the 30mer peptide sequence on day 0 and a cocktail of MHC Class I peptides (peptides 4, 5 and 6 from Table 1) on day 15 with IFA or CpG ODN 1826, individually or in combination. A cocktail of MHC Class I peptides was used as a boost in order to promote the expansion of $CD8^+$ T-cells specific for these peptides. Seven days after the last immunisation, mice were culled and the responsiveness of splenocytes against all the predicted HLA-A2 and HLA-DR1 peptides within the HAGE 30mer

sequence was assessed using an IFN-γ ELISpot assay (MabTech, cat #3321-1H-6). The results of the *ex vivo* assays (Figure 1) show that the HLA-A*0201 restricted peptides 4 ($HAGE_{297-305}$) and 6 ($HAGE_{296-305}$) and the HLA-DRB*0101 restricted peptide 7 ($HAGE_{291-305}$) induced the greatest peptide-specific IFN-γ responses amongst all of the HLA-A2 and DR1 epitopes tested. Interestingly, despite the high similarity between the sequences of peptide 4, 5 and 6, peptide 4 and 6 induced higher numbers of IFN-γ secreting cells. Combining IFA and CpG significantly enhanced the peptide-specific IFN-γ response (Figure 1).

Peptide versus DNA vaccine (ImmunoBody®)

The ImmunoBody®-HAGE construct was designed to carry the HAGE 30mer ($HAGE_{287-316}$) peptide and this was inserted in the heavy variable region.

HHDII/DR1 mice were immunised with either ImmunoBody®-HAGE or HAGE 30mer peptide/IFA+CpG vaccine. Seven days after the last immunisation, splenocytes from immunised animals were harvested and responsiveness to the HLA-A2 and DR1 peptides derived from the HAGE 30mer sequence listed in Table 1 were assessed *in vitro* using an IFN-γ ELISpot assay.

Figure 2a demonstrates the superiority of the ImmunoBody®-HAGE vaccine in generating a specific immune response against the assessed peptides, as shown by the significantly higher response (in the context of the number of cells secreting IFN-γ) against peptides 5, 6 and 7. Interestingly, immunisation of mice with the HAGE 30mer peptide followed by a boost using a cocktail of MHC Class I peptides generated a significant number of IFN-γ secreting cells in response to peptide 4, a response which was not observed when animals were immunised using the ImmunoBody®-HAGE. In contrast, strong immune responses against peptide 5 were observed (Figure 2). Immune responsiveness against peptide 6 was detected in both immunisation strategies. These findings indicate that peptide 4 was not naturally endogenously processed, despite being predicted to have a higher binding affinity for HLA-A2 molecules.

The ImmunoBody®-HAGE vaccine was also better at generating high avidity T-cell responses than the peptide/IFA+CpG vaccination strategy (Figure 2b, c).

Induction of HAGE-specific cytotoxicity

The ability of immunisation strategies to trigger cytotoxic T cells having the capacity to recognise and kill peptide-pulsed target cells and TNBC cells naturally expressing HAGE was then assessed.

As a model system, the murine melanoma cell line B16F1 (C57BL/6 background) was knocked out for both murine endogenous MHC class I and II by Zinc finger Nuclease (ZFN) technology and stably transfected with plasmids to express both HHDII and HLA-DR1 molecules. Thereafter, the resultant cells were transduced with HAGE-encoding viral

construct and transfected with the Luc2 encoding plasmid vector. HAGE expression was confirmed at both mRNA (Figure 3a) and protein levels (Figure 3b, c). HHDII, DR1 expression (Figure 3d) and luciferase reporter gene expression (Figure 3e) of the B16/HHDII/DR1/HAGE, Luc2 cells were assessed by flow cytometry and luciferase reporter assays respectively.

Splenocytes from mice immunised with either HAGE 30mer peptide vaccine or ImmunoBody®-HAGE DNA vaccine were cultured for 1 week with the MHC Class I peptide cocktail (peptide 4-LMPGFIHLV, 5-YLMPGFIHL, 6-YLMPGFIHLV), after which the ability of these cells to recognise and kill peptide-pulsed or HAGE-expressing target cells was assessed using IFN-γ ELISpot and ^{51}Chromium release cytotoxicity assays. Splenocytes co-cultured with target cells (T2 ± peptide, B16/HHDII/DR1$^{HAGE+/-}$ cells MDA-MB-231$^{HAGE+/HLA-A2+}$, and MDA-MB-468$^{HAGE+/HLA-A2-}$) in an IFN-γ ELISpot plate showed HAGE-specific cytokine release in a peptide and HLA-A2 restricted manner (Figure 4a). These cells were also able to specifically kill peptide-pulsed T2 (Figure 4b) cells, and B16 cells expressing HAGE (Figure 4c). The ImmunoBody®-HAGE DNA vaccine induced significantly higher cytotoxicity against B16/HAGE^{+} tumour cells than the HAGE peptide vaccine. Moreover, the cytotoxicity of ImmunoBody®-derived T cells against human MDA-MB-231 and MDA-MB-468 TNBC cells was also assessed as they also express HAGE (Figure 3f, g). Figure 4d demonstrates the capacity of the ImmunoBody®-HAGE vaccine to generate immune cells with the ability to specifically target HAGE-expressing and HLA-A2^{+} TNBC cells. The cytotoxicity against MDA-MB-231 (HLA.A2^{+}) cells was significantly higher than the cytotoxicity against MDA-MB-468 (HLA.A2^{-}) cells.

Pre-immunisation with ImmunoBody®-HAGE vaccine delays the growth of implanted B16/HHDII/DR1/HAGE/Luc cells in both subcutaneous and metastasis settings

Having demonstrated the superiority of the ImmunoBody®-HAGE over HAGE-30mer peptide, only the HAGE-ImmunoBody® was used in tumour model experiments. In these, the ability of the ImmunoBody®-HAGE vaccine to prevent/delay the growth of HAGE-expressing B16/HHDII/DR1/HAGE/Luc tumours after either subcutaneous implantation or intravenous injection (as an experimental model of metastasis) into HHDII/DR1 mice was tested in both prophylactic and therapeutic settings.

In prophylactic studies, HHDII/DR1 mice were immunised on day 0, 7 and 15, and 7 days after the last immunisation (Day 22) animals were challenged with B16/HHDII/DR1//HAGE/Luc tumour cells. Control mice received only tumour cells as shown in supplementary figure 2. Figure 5a shows representative images of tumour-bearing mice from different experimental groups. It was observed that by day 34 after tumour cell injection, 3/11 mice from the prophylactic group of the subcutaneous model remained alive (Figure 5e), whereas none of the mice from the control and therapeutic groups were alive at that point. Figure 5b-d shows that immunisation with ImmunoBody®-HAGE vaccine significantly delayed tumour uptake and growth. The ability of the vaccine to slow/eradicate established tumour growth was assessed by first implanting B16/HHDII/DR1//HAGE/Luc cells and then starting vaccination two days later. Figure 5e shows that the

vaccine was unable to delay the growth of established tumours in this setting. The anti-tumour efficacy of ImmunoBody®-HAGE vaccine was also tested in an experimental metastatic model. A similar anti-tumour protection with significant delay in the onset of tumour growth (Figure 6b–d) was observed in immunised mice compared to non.immunised mice groups. Mice administered with vaccine before tumour injections showed prolonged survival compared to non-immunised mice and mice that received therapy (Figure 6e). While such model of experimental metastasis does not, however, adequately mimic human metastatic breast cancer because the cells injected systemically directly go to the metastasis site without first establishing primary disease elsewhere, it is the only model available to assess the efficacy of a vaccine in HHDII/DR1 mice.

Prophylactic vaccinations were then combined with therapeutic vaccinations to assess whether the continued vaccination, even after the appearance of tumour, could improve efficacy and outcomes.

Tumour growth was significantly further delayed when vaccination was continued after the administration of a lethal dose of B16/HHDII$^+$/DR1$^+$/HAGE$^+$ tumour cells (Figure 7a–c). However, the addition of anti-PD1 antibody did not further improve this outcome (Figure 7d, e). Interestingly, we have found that more than 60% of all tumour-infiltrating T-cells were PD1$^+$, whereas < 20% of the T-cells located in the spleen of the same animals express PD-1 [24]. Hence, to understand association of TILs and additional mechanisms of T cell suppression B16/HDII/DR1/HAGE, Luc2 tumour-derived TILs were profiled using multi-parameter flow cytometry. For these studies, mice were grouped according to their tumour size/weight (Figure 8a). Five mice injected with tumour cells and receiving sham vaccinations and isotype control for the anti-PD1 antibody were used as comparators. Despite a significant difference in the weight between small and large tumours group, no difference in the percentage of macrophages (CD68$^+$/F4/80$^+$) infiltrating small and large tumours was detected (Figure 8b). However, a significant percentage of these macrophages also expressed CD206 and IL-10, features that are associated M2 macrophages, whereas the percentage of macrophages negative CD206 and IL-10 expression but expressing TNFα, was significantly higher in mice bearing small tumours. In addition, larger tumours had more Treg (CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$) cells than smaller tumours, whereas the proportion of CD3$^+$CD4$^+$ T-cells was not significantly affected (Figure 9a). No differences in the proportion of myeloid suppressive cells (CD11b$^+$/LY-6C$^+$/LY-6G$^{+/-}$) was found (Figure 9b). Moreover, TILs extracted from mice which did not receive the vaccine contained a significantly higher proportion of 'exhausted' (CD3$^+$/CD8$^+$/PD1$^+$/Tim3$^+$) CD8$^+$ T-cells (Supplementary figure 1).

DISCUSSION

Recent years have seen remarkable advances in the field of cancer immunotherapy. Active immunotherapy mainly focuses on eradication of cancerous cells by induction of T-cell-mediated anti-tumour responses. Antigens that are differentially expressed or are selectively induced/arise in tumour tissues, and which can trigger anti-tumour immune responses, represent potential immunotherapeutic targets. The induction of potent anti-tumour immune responses is critically dependent on the choice of target antigen and although tumour associated antigens are easier to identify and are shared by many patients, vaccines based on these have not yet been proven to be effective in clinical trials. In addition, as they are

not necessarily tumour-specific, there is the potential for severe adverse events due to off target effects. In contrast, neoepitopes derived from mutated antigens are tumour-specific, more immunogenic and also patient-specific. Cancer testis antigens (CTAs) represent a compromise between these two categories of antigens due to their restricted expression pattern, almost exclusively in tumour, with little to no normal tissue expression (except for testis and placenta) and are, when combined with an appropriate adjuvant/delivery system, immunogenic.

HAGE is a CTA (CT13) which was first identified by Martelange *et al*. and found to be expressed 100-fold higher in tumour tissues than in normal tissues, except testis [26]. HAGE mRNA expression in many malignant tumours along with the expression of BAGE, MAGE, GAGE indicate the potential role of CTAs as targets and/or diagnostic markers [27]. HAGE has also been shown to promote the proliferation of malignant cells and it has also been shown to be involved in the process of tumorigenesis in multiple cancer types [28]. HAGE has also been shown to be expressed in 43% of locally advanced primary TNBC tumours before anthracycline combination Neo-ACT. Following anthracycline combination Neo-ACT, a pCR (pathological Complete Response) was achieved in 48% of $HAGE^+$ tumours, but in only 14% (8/56) of $HAGE^-$ TNBC tumours [4].

Although HAGE protein has previously been shown to be immunogenic, no specific peptide region responsible for triggering and driving immunogenicity was identified [29]. Melief *et al*. have previously demonstrated that the use of long peptide fragments (15-30) rather than the entire protein is better at inducing $CD8^+$ T-cells. Therefore, in this current study, an immunogenic region within HAGE protein has been identified and its potential to generate specific immune responses against tumours expressing HAGE investigated. The study began with the identification of a 30 amino-acid sequence predicted to encompass several HLA-restricted immunogenic epitopes by *in silico* SYFPEITHI analysis. Splenocytes derived from HAGE-derived 30mer (either peptide with CpG/IFA or as ImmunoBody® DNA construct) vaccinated HHDII/DR1 mice were able to induce strong IFN-γ responses. Our results showed that although the combination of peptide and CpG/IFA was better than peptide with either adjuvant alone, ImmunoBody®-HAGE was significantly better at generating an IFN-γ response. The efficiency of DNA vaccines to deliver antigenic epitopes within a human antibody IgG1 scheme has previously been demonstrated for other antigens [10, 11]. Among the short peptides, peptide 5 (YLMPGHFIL) and peptide 6 (YLMPGFIHLV) which only differ from each other by an additional valine residue, both HLA-A*0201-restricted peptides and peptide 7 (GKTCLYLMPGHFILV) that encompasses both peptide 5 and peptide 6 were all able to generate strong immune responses, as assessed by the high number of IFN-γ producing cells. Interestingly, this region is also predicted to include an HLA-DR4 T-cell epitope, which suggests a certain HLA promiscuity of this sequence.

More importantly, splenocytes from animals immunised with the ImmunoBody®-HAGE vaccine recognised and responded to HAGE-expressing target cells. Indeed, a significantly higher number of vaccine-induced splenocytes co-cultured with T2 cells pulsed with HAGE-derived peptides or B16/HHI/DR1/$HAGE^+$/Luc cells produced IFN-γ than when

co-cultured with non-HAGE expressing/pulsed target cells. Furthermore, vaccine-induced immune cells were also able to kill $HAGE^{+/pulsed}$ target cells, as assessed by *in vitro*.[51] Chromium release cytotoxicity assays. These results confirmed the superior immunogenicity of the ImmunoBody®-HAGE vaccine.

The *in vivo* anti-tumour efficacy of this vaccine was then confirmed by *in vivo* tumour challenge studies using HHDII/DR1 mice models in both prophylactic and therapeutic settings. Immunisation with the ImmunoBody®-HAGE vaccine significantly delayed the growth of both subcutaneous and i.v. injected B16/$HHDII^{+}$/$DR1^{+}$/$HAGE^{+}$ tumours. However, the vaccine on its own was not able to influence the growth of tumour cells when these were injected first (therapeutic settings).

Murine B16 melanoma cells are aggressive cells that grow quickly once injected *in vivo* and by the time the vaccine generated a strong immune response the now well-established tumour cells created an immunosuppressive microenvironment. In addition, 100% of B16 cells express PD-L1 molecules which will bind to the PD1 expressed by activated T-cells and induce their death. We have previously shown that over 80% of TILs express PD-1 while T-cells found in the spleen of the same animals do not [25].

The use of immune checkpoint inhibitors can also have 'adjuvant' effects by enhancing the efficacy of the vaccine, as has been demonstrated in preclinical models combining SCIB1 and SCIB2 ImmunoBody® vaccines with anti-PD1 [30, 31]. Interestingly, the schedule chosen to administer both checkpoint inhibitor and vaccine will influence the efficacy of the vaccine. Indeed, although the efficacy of both the GVAX prostate specific antigen (PSA) prostate cancer vaccine and the TG4010 (Muc-1) vaccine for advanced non-small cell lung cancer vaccine was improved by administering anti-CTLA-4 and anti-PD-1 respectively several times after vaccinations, the PSA-targeted DNA vaccine was found to be most effective if given at the same time as the anti-PD-1 [32-34].

We propose that the future clinical potential for this vaccine will involve its administration to patients with TNBC patients who have received conventional treatment(s) and have been declared tumour free, given that relapse is known to occur in some of these patients within 2 years following the end of their treatment. Immunised mice were therefore challenged with a lethal dose of tumour cells and additional vaccinations with or without the addition of anti-PD1 administered. Our results demonstrate that the additional vaccinations significantly prolonged the time to death compared to prophylactic setting, but that anti-PD-1 had no influence on this prolongation of survival. We hypothesised that additional suppressive mechanism(s) must be operating and therefore profiled TILs using flow cytometry. Our results clearly show that M2 ($CD68^{+}$/$F4/80^{+}$/$CD206^{+}$/$IL\text{-}10^{+}$) macrophages predominate in large tumours and also that large tumours were more highly infiltrated by Treg ($CD3^{+}$/$CD4^{+}$/$CD25^{+}$/$FoxP3^{+}$) cells. No differences were found in the level of MDSCs ($CD11b^{+}$/$LY6C^{+}$/$LY6G^{+}$) between the two tumour types.

In conclusion, our studies indicate that the HAGE antigen is immunogenic and the 30mer peptide region within HAGE is efficient at generating high frequencies of high-avidity T cells that can induce potent anti-tumour responses against HAGE expressing tumours. The ImmunoBody®-HAGE vaccine could therefore be translated into the clinic for the treatment of patients with TNBC expressing HAGE after they have received chemotherapeutic treatment with the intention of preventing relapse. Targeting additional antigens as well as including approaches to re-direct M2 macrophages towards M1 macrophages might further enhance the therapeutic effect. Moreover, it is possible that patients with TNBC with no/few TILs ('immune desert') would benefit from first receiving an antigen-specific vaccine (such as ImmunoBody®-HAGE) to induce a strong and, at least partially, protective anti-tumour immunity followed by additional therapies aimed to alleviate immunosuppressive effect induced by the tumour cells.

METHODS

Animals

HHDII-DR1 double transgenic (Tg) mice expressing human α1 and α2 chains of HLA-A*0201 chimeric with the α3 chain of the H-2Dd allele (HHDII), also expressing HLA-DRB*0101 and knocked out for the expression of murine MHC class I (H-2b) and II (I-Ab) were provided by Dr. Lone (CNRS, Orleans, France). The use of protocols and procedures in this study and animal care were employed in accordance with EU Directive 2010/63/EU and UK Home Office Code of Practice for the housing and care of animals bred, supplied or used for scientific purposes. Experiments to assess the vaccine immunogenicity were performed at least once with three female mice 6-8 weeks of age. A minimum of ten mice per test group were used for the *in vivo* tumour growth models.

Cell lines

The TAP deficient T2 cell line which is HLA-A2 lymphoblastoid suspension cells was a gift from Dr. J. Bartholomew (Paterson Institute, Manchester, UK). The human TNBC cell lines, MDA-MB-231 and MDA-MB-468 were purchased from ATCC and cultured in Leibovitz (L15) medium supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v L-Glutamine.

The murine melanoma cell line B16F1, which was knocked out for both murine endogenous MHC class I and II by Zinc finger Nuclease (ZFN) technology and stably transfected with plasmids to express both HHDII and HLA-DR1 molecules (hereafter referred to as B16/HHDII/DR1), was a generous gift from Prof. Lindy Durrant (Faculty of Medicine and Health Sciences, Nottingham University and Scancell Ltd). These cells were grown in RPMI 1640 supplemented with 10% v/v FBS, 1% v/v L-Glutamine, 300 μg mL^{-1} Hygromycin and 500 μg mL^{-1} Geneticin.

Identification of immunogenic and MHC binding predictions

The SYFPEITHI database algorithm was used to identify an immunogenic HAGE-derived sequence (30mer) based on the presence and position of anchor residues that influence their binding affinities to different HLA alleles [HLA-A*0201

(HLA-A2) and HLA-DRB*0101 (HLA-DR1)]. Table 1 summarises peptides derived from the 30mer and their binding scores to different HLA-haplotypes.

ImmunoBody®-HAGE DNA vaccine

ImmunoBody® vaccines are designed to generate potent T cell responses capable of a broad anti-tumour effect. They are DNA vaccines that encode a protein in the form of an antibody, but the parts of the antibody that would normally bind to the target protein are replaced with epitopes from a cancer antigen [10, 11]. The ImmunoBody®-HAGE vaccine incorporates the cDNA sequence for the HAGE 287-316 (QTGTGKTLCYLMPGFIHLVLQPSLKGQRNR) 30mer peptide region inserted into the CDRH2 heavy variable of the human IgG1 chain alongside the light human Kappa chain encoded within the double expression vector pDCOrig. The ImmunoBody® heavy and light chains are within separate expression cassettes, both of which are under control of a CMV promoter, as described [10, 11].

Peptides

Peptides to be used for immunisations and *ex vivo* analyses were synthesised by GenScript™ (Piscataway, USA) and reconstituted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at 10 mg mL^{-1} concentration and stored at -80°C.

Animal immunisation

For administration of DNA vaccines, a Helios™ gene gun (Bio-Rad Laboratories) was used. For this, DNA bullets were prepared by coating 1µg gold particles with plasmid ImmunoBody® DNA and were tested for correct dispersal of the DNA prior to intradermal administration into HHDII/DR1 transgenic mice on days 1, 8 and 15.

For peptide immunisations, 100µL of the formulations were prepared with 75µg of peptide in IFA (50 µL per mouse, Sigma-Aldrich) or CpG-ODN (10 mg mL^{-1}, 50 µg per mouse, Eurofins Scientific). Peptide vaccines were subcutaneously administered into HHDII/DR1 mice on day 1 and day 15 at the base of the tail. For both DNA and peptide vaccinations, spleens were harvested to isolate splenocytes for the *ex vivo* assays one week after the final immunisation.

Isolation of splenocytes

Spleens were excised from naïve or vaccinated HHDII/DR1 mice. Thereafter, spleens were flushed out using T cell medium (RPMI 1640, Life Technologies / Thermo Fisher Scientific, containing 1% w/v L-Glutamine (SLS/Lonza), 10% v/v FBS (Fisher/GE Healthcare), 2% v/v HEPES (SLS /Lonza), 0.1% v/v Fungizone (Promega), 0.5% v/v β-mercaptoethanol (Sigma-Aldrich)), after which they were passed through a strainer to remove tissue debris and centrifuged at 300*g* for 10 min. Cell pellets were resuspended in T cell medium for counting and plating.

In vitro stimulation of the murine splenocytes

Splenocytes obtained from mice spleens were stimulated *in vitro* at 4 x 10^6 mL^{-1} with Class I (1 µL mL^{-1}) peptide cocktail (contains three Class I peptides, 0.33 µg of each peptide mL^{-1}). Cells were stimulated *in vitro* in the presence of β-mercaptoethanol (0.02 mM) and IL-2 (50U mL^{-1}) at 37°C, 5% CO_2 for 7 days, followed by cell harvesting and counting for plating with target cells for cytotoxicity assays.

Vaccine responses: ELISpot assays

The frequency of HAGE 30mer responsive cells after 48 h of *in vitro* stimulation was determined using a murine IFN-γ ELISpot kit (Mabtech, Cat #3321-2A). For this, transmembrane 96-well Millipore plates were pre-coated with capture antibody according to the manufacturer's protocol. For each experiment, 0.5 x 10^6 splenocytes per well were plated in triplicate with 1 µg mL^{-1} and 10 µg mL^{-1} of MHC class I (HLA-A*0201, 8-10mer) and class II (HLA-DRB*0101, 15mer) peptides respectively. Experimental results are presented as the number of IFN-γ spot-forming units (SFUs) *versus* control wells that contain medium alone. The spots were counted using an ImmunoSpot ELISPOT analyser (Cellular Technology Limited, CTL).

Isolation of $CD8^+$ T cells from spleens

$CD8^+$ cells were isolated from spleens derived from vaccinated HHDII/DR1 mice using a Dynabeads Untouched Mouse $CD8^+$ Cells Kit (Invitrogen *Cat* #1417D) according to the manufacturer's protocol. At least 50 x 10^6 splenocytes were used to obtain pure and untouched $CD8^+$ target cells. The purity of the isolated cells was determined by flow cytometry and was >80% (data not shown).

Construction of plasmid for HAGE transfections

The entire cDNA sequence of the HAGE gene (comprising full-length of 1959 base pairs) along with Kozak sequence and ATG start codon at 5' end was inserted into the pUC57-Kan cloning vector and synthesised by GenScript™. The HAGE gene was then successfully cloned into pLenti-Puro (Addgene) to generate pLenti/HAGE. RNA and protein expression were confirmed by the analysis of transiently transfected HEK293 cells. Plasmid DNA was purified using a Qiagen DNA Extraction kit (Qiagen).

Transfection of B16/HHDII/DR1 cells with the Luciferase (Luc2) reporter gene

B16/HHDII/DR1 were then further transfected with pBUD plasmids encoding the Luciferase (Luc2) reporter genes using Lipofectamine 3000, according to manufacturer's protocol (Invitrogen / Thermo Fisher Scientific). After 24 h, transfected B16/HHDII/DR1/Luc2 cells were selected by culturing in RPMI containing 550 µg mL^{-1} zeocin.

Viral transduction of B16/HHDII/DR1/Luc2 cells to express HAGE

B16/HHDII/DR1DR1/Luc2 cells were induced to express HAGE by transduction using viral particles generated from HEK293T cells transfected pLenti/puro encoding HAGE gene along with packaging plasmid and envelope plasmid at

4:2:1, respectively using Lipofectamine 3000. HEK293T supernatants collected after 24 h were used to transduce target cells with transduction medium (50% v/v culture medium / 50% v/v viral supernatant / 8 μg mL^{-1} hexadimethrine bromide). Cells were incubated with viral particles for 18–20 h at 37°C and selected by culturing in fresh medium containing 1 μg mL^{-1} puromycin.

HAGE expression: Immunofluorescence

Adherent cells cultured on coverslips were washed with phosphate-buffered saline (PBS) prior to fixation and permeabilization using 1% w/v paraformaldehyde for 20 min at 4°C. Cells were washed twice with PBS and incubated in blocking buffer (5% w/v bovine serum albumin (BSA) in 0.05% v/v Tween in PBS) for 1 h. Cells were then stained with 20 μg mL^{-1} of anti-DDX43 (HAGE) primary antibody (Sigma-Aldrich, cat #HPA031381) overnight in blocking buffer and washed twice in wash buffer (0.05% v/v Tween in PBS) prior to 1 h of incubation with 10 μg mL^{-1} of Alexa Fluor™ 594-conjugated goat anti-rabbit secondary antibody in blocking buffer (Thermo Fisher Scientific, cat. # A-11012). Coverslips were washed twice with wash buffer and dried at 37°C, before mounting onto slides for imaging using a Leica fluorescence microscope.

HAGE expression: Real-time PCR

cDNA synthesised from the 2μg of mRNA samples were used for qRT-PCR using SYBR™ green, fluorescent dye (Invitrogen / Thermo Fisher Scientific). Thermocycling reactions were set up in PCR tubes containing 1 μL of cDNA template, 4.75 μL of SYBR™ Green supermix and 5pmol (0.5 μL) of each of the specific primers. qRT-PCR was setup for 40 cycles of denaturation (95°C for 10 s), annealing (58°C for 18 s) and extension (72°C for 20 s) and stop at 4°C. (HAGE primers Forward: CCACATGCACTTTCGACGAT, Reverse: ATTCCTGGTCGGTTCCTCTG). GUSB house-keeping gene was used as a control (Forward: ACTGAACAGTCACCGAC, Reverse: AAACATTGTGACTTGGCTAC).

HAGE expression: Western blotting

Cell lysates were prepared by re-suspending 1 x 10^6 cells in 100 μL of Laemelli buffer and boiling at 95°C for 15 min. 20 μg of lysate was loaded onto a 10% w/v SDS PAGE gel. After overnight transfer of proteins onto PDVF membrane and blocking with 5% w/v milk powder, membranes were incubated with anti-human HAGE primary antibody (anti-DDX43, Sigma-Aldrich, cat #HPA031381, B actin, Sigma-Aldrich, cat #A5441) and then with anti-rabbit or mouse (HRP) secondary antibody (Cell Signalling Technology, cat #7074, cat #7076) for 1 h to enable protein detection using an ECL chemiluminescence kit (Bio-Rad Laboratories).

Effect of immunisation on anti-tumour immunity: in vitro cytotoxicity assays

Naïve splenocytes were matured by using 25 μg mL^{-1} Lipopolysaccharide (LPS, Sigma-Aldrich) and dextran sulphate (0.7 μg mL^{-1}, Sigma-Aldrich) for six days, after which they were pulsed with HAGE-derived Class I peptides (1 μg mL^{-1}). Mature LPS cells pre-treated with mitomycin (1 μg per 10^6 cells) were then pulsed for 90 min with 10 μg mL^{-1} of 30mer

peptide. The peptide-pulsed LPS blast cells were co-cultured with splenocytes from immunised mice for a week after which they were harvested to perform ^{51}Chromium release killing assays.

Effect of immunisation on anti-tumour immunity: Prophylactic and therapeutic tumour studies (a schematic representation of the various immunisation procedure can be found in Supplementary figure 2)

For the tumour growth studies, HHDII/DR1 mice were divided into 3 groups (10 mice per group).

For prophylactic studies, mice were immunised with the ImmunoBody®-HAGE vaccine on day 0, 7 and 15, and on day 22 they were implanted with 0.75 x 10^6 B16/HHDII/DR1/HAGE/Luc2 cells subcutaneously.

For therapeutic studies, mice were implanted with 0.75 x 10^6 B16/HHDII/DR1/HAGE/Luc2 cells subcutaneously, and 2 days later received the ImmunoBody®-HAGE vaccine followed by two additional vaccinations on day 7 and 14 post-tumour implantations.

The control group were implanted with 0.75 x 10^6 B16/HHDII/DR1/HAGE/Luc2 cells subcutaneously and monitored for tumour growth.

Tumour growth was monitored using callipers ([Volume = $(\pi/6)$*(diameter$_{long}$)*(diameter$_{short}$)^2]) and bioluminescence *in vivo* imaging following the administration of 150 mg kg^{-1} D-Luciferin (using Perkin Elmer IVIS® Lumina Series III) until animals exhibited clinical signs and/or loss of > 15% body weight loss, or tumours reached a maximum mean diameter of 1.2 cm^2 (control and prophylactic groups) or 1.5 cm^2 (therapeutic group).

Effect of immunisation on anti-tumour immunity: Experimental Metastasis

The ability of the ImmunoBody®-HAGE vaccine to control the growth of i.v injected B16/HHDII+/DR1+/HAGE+/Luc+ cells was also determined (3 animal groups, 11 animals in each group). For this, the HHDII/DR1 mice were intravenously administered with 0.75 x 10^6 B16/HHDII/DR1/HAGE/Luc2 cells. For the prophylactic study, mice were immunised with the ImmunoBody®-HAGE vaccine on days 0, 7 and 15, and on day 7 mice were rechallenged with 0.75 x 10^6 of B16/HHDII/DR1/HAGE/Luc2 cells intravenously. For therapeutic studies, mice received 0.75 x 10^6 of B16/HHDII/DR1/HAGE/Luc2 cells i.v on day 0 and the ImmunoBody®-HAGE vaccine on days 2, 9 and 16 thereafter. Tumour growth and spread were monitored by luminescent *in vivo* imaging following the administration of 150 mg kg^{-1} D-Luciferin. Mice were culled as soon as they exhibited clinical signs and/or bodyweight loss > 15%.

Extraction of TILs from B16 HAGE tumours

Mice were culled by cervical dislocation and tumours were extracted and placed in T-cell medium. Tumours were placed in a petri dish with 5 mL of medium and finely minced using a scalpel blade. The resultant cell suspension was transferred into a 15-mL Falcon tube, to which were added DNAse (Sigma) and collagenase I at a concentration of 50 µg mL^{-1} and 0.1 U mL^{-1} respectively. The resulting cell suspension was then incubated at 37°C in an orbital incubator at 200 RPM for 1 h. After incubation, the cell suspension was passed through a 40 µM cell strainer and clumps were pushed through using a

syringe plunger and washed through with 10 mL of fresh medium. Cells were then pelleted and resuspended in 3.5 mL of medium, this 3.5 mL of medium was layered over 7 mL of Ficoll™ Paque Plus. The cells were then spun at 800*g* for 20 min with no brakes. The resulting cells from the interface were taken and resuspended and counted using a NucleoCounter NC250 (Chemometec). The CD45 cells were then isolated using CD45 TIL microbeads (Miltenyi Biotec) following the manufacturer's protocol. In brief, 10 µL of CD45 TIL microbeads were added per 10^7 cells and incubated for 15 min at 4°C. Isolation buffer (degassed PBS + 0.5 % w/w BSA + 2 mM EDTA) was then added to a final volume of 500 µL. A MACS™ magnetic separation column (Milteny Biotec) was prepared by placing the column in a magnet and running 500 µL of isolation buffer through the column. After isolation buffer was passed through the column, the 500 µL of labelled cell suspension was run through the column. Labelled cells are captured and the column was washed three times using 500 µL isolation buffer to remove any residual unlabelled cells. The column was then removed from the magnet and 1 mL of separation buffer was added, labelled cells were then flushed using a plunger pressed down the column. The resulting cells were then counted and transferred to polystyrene flow cytometry tubes for subsequent analysis.

Flow cytometric analysis of TILs

The different cell subsets were identified by flow cytometry using the following combinations of conjugated monoclonal antibodies:

M1/M2 Macrophages

PE conjugated anti-F4/80 (clone BM8, cat # 123110),; PE/Dazzle™ 594 conjugated anti-CD80 (clone 16-10A1, cat # 104738),; APC conjugated anti-CD206 (clone C068C2, cat # 141708),; Alexa Fluor™ 700 conjugated anti-CD86 700 (clone GL-1, cat # 105024),; APC-Cy7™ conjugated anti MHC class II; FITC conjugated anti- (clone L243, cat # 307618),IL-10 (clone JES5-16E3, cat # 505006),; PerCP-Cy5.5 (clone FA-11, cat # 137010), ™ conjugated anti-CD68; Brilliant Violet 421™ conjugated anti-TNFα (clone MP6-XT22, cat # 506328) (all from Biolegend), Arginase 1 PE-Cyanine7 (eBioscience™, clone A1exF5, cat # 25-3697-82) and LIVE/DEAD™ Fixable Yellow Dead Cell Stain (Invitrogen, cat # L34959).

Regulatory T-cells

efluor™ 660 conjugated anti-FoxP3 (eBioscience™, clone FJK-16s, cat # 50-5773-82),); PerCP-Cy5.5™ conjugated anti-, cat # L34959CD25 (clone 3C7, cat # 101912),; Alexa Fluor™ 700 conjugated anti- CD4 (clone GK1.5 cat # 100430),; APC-Cy7™ conjugated anti-CD8a (clone 53-6.7, cat # 100714),; Brilliant Violet 421™ conjugated anti-CD3 (clone 17A2, cat # 100228) (all from Biolegend) and Live/dead yellow); LIVE/DEAD™ Fixable Yellow Dead Cell Stain (Invitrogen, cat # L34959).).

Activated/exhausted T-cells

FITC conjugated anti-GITR (clone DTA-1, cat # 126308),; PE conjugated anti-OX40 (clone OX-86, cat # 119410),; PE-efluor™ 610 conjugated anti-CTLA-4; (clone UC10-4B9, cat # 106318), PerCP-Cy5.5 (clone C9B7W, cat # 125212),™ conjugated anti-LAG3; PE-Cy7 (clone RMT3-23, cat # 119716),™ conjugated anti-TIM3; APC conjugated anti-PD-1 APC (clone 29F.1A12, cat # 135210); Alexa Fluor™ 700 conjugated anti-CD4; (clone GK1.5 cat # 100430), APC-Cy7™ conjugated anti-CD8a; (clone 53-6.7, cat # 100714), Brilliant Violet 421™ conjugated anti-CD3 (clone 17A2, cat # 100228) (all from Biolegend) and LIVE/DEAD™ Fixable Yellow Dead Cell Stain (Invitrogen), cat # L34959).

Myeloid derived suppressor cells (MDCs)

FITC (clone QCRL-3, cat # 12760), conjugated anti-Ly6G; PE-conjugated anti-CD11b-PE (clone M1/70, cat #:101207), Ly6C; APC conjugated anti-Ly6c (clone HK1.4, cat # 128015) (all from Biolegend) and LIVE/DEAD™ Fixable Yellow Dead Cell Stain (Invitrogen), cat # L34959).

Flow cytometric analysis of splenocytes from tumour-bearing mice

Mice were culled by cervical dislocation and spleens were extracted and placed in T-cell medium. Splenocytes were extracted as described and 1 x 10^6 cells were transferred to the wells of a 96-well U-bottomed plate. Cells were then stimulated with 1 µg mL^{-1} class I peptides, 10 µg mL^{-1} class II peptide, a combination of class I and II peptides (0.5 µg mL^{-1} of each class I peptide and 10 µg mL^{-1} of the class II peptide) or no peptide with 1 µg mL^{-1} of monoclonal antibodies against CD28 (clone 37.51, cat # 553294) and 1 CD49d (clone 9C10, cat # 553314) (both from BD). Cells were incubated for 1 h at 37°C in a humidified tissue culture incubator (Sanyo). After incubation, cells were treated with brefeldin A and monensin (both from Biolegend) and cells were stained using a FITC conjugated antibody against CD107a-FITC (Biolegend, clone 1D4B, cat # 121606) for 5 h at 37°C in a humidified tissue culture incubator (Sanyo). After 5 h, cells were removed from the 96-well plate and transferred to 12x75 mm tubes. Cells were then washed with 2 mL of PBS by centrifugation at 300*g* for 5 min. After washing cells were re-suspended in 50 µL of FCS and 0.5 µg uL^{-1} of FcR blocking reagent (anti-CD16/32, Biolegend, clone 93, cat # 101302) was added to each tube. The tubes were then incubated at 4°C for 15 min, after which they were incubated with Alexa Fluor™ 700 conjugated anti-CD4 (clone GK1.5 cat # 100430), APC-Cy7™ conjugated anti-CD8a (clone 53-6.7, cat # 100714),, Brilliant Violet 421™ anti-CD3 monoclonal antibodies (clone 17A2, cat # 100228) (all from Biolegend) and LIVE/DEAD™ Fixable Yellow Dead Cell Stain (Invitrogen), cat # L34959 at 4°C for 30 min.

After incubation cells, were incubated with 25 µL of fixative reagent R1 (Beckman Coulter Fix-Perm kit) for 15 min at 4°C. After fixation cells were resuspended in 300 µL permeabilising reagent R2 (Beckman Coulter FixPerm kit) and incubated with PE-conjugated anti-TNF-α; (clone MP6-XT22, cat # 506306), PerCP-Cy5.5 (clone JES6-5H4, cat # 503822),™ anti-IL-2; PE-Cy7™ conjugated anti-IFN-γ; (clone XMG1.2, cat # 505826), APC conjugated anti-Granzyme B-APC (clone QA16A02, cat # 372204) (all from Biolegend) and PE-efluor™ 610 conjugated anti-Ki67 (eBioscience, clone SolA15, cat # 61-5698-80) monoclonal antibodies. Tubes were then incubated at 4°C for 30 min. After incubation

cells were washed in 2 mL of reagent R3 (Beckman Coulter Fix-Perm kit) via centrifugation at 300*g* for 5 min. After washing stained cells were re-suspended in 250 μL of reagent R3 and analysed via flow cytometry using a Beckman Coulter Gallios™ flow cytometer.

Statistical analysis

Data were analysed with GraphPad Prism8 software. The Mann-Whitney *U*-test was used for comparison of two non-parametric datasets and one-way ANOVA or two-way ANOVA tests with post hoc testing using Tukey's multiple comparison were used for multigroup comparisons. *P*-values < 0.05 were considered significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). All data are presented as Mean ± SEM.

ACKNOWLEDGMENTS

We thank members of the John van Geest Cancer Research Centre for their thoughtful discussions regarding this work. This study was supported by the John and Lucille van Geest Foundation, Nottingham Trent University A03 QR Allocation and a generous donation from Mrs. Shirley Chan.

CONFLICTS OF INTEREST

LD is a Director of Scancell Ltd and VB and RM are employees of Scancell Ltd. ImmunoBody® is a registered trademark of Scancell Ltd. AGP is the CEO of multimmune GmbH, Munich, Germany.

REFERENCES

1 Ferlay J, Colombet M, Soerjomataram I, *et al.* Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur J Cancer* 2018; **103:** 356-387.

2 Abramson VG, Mayer IA, Molecular heterogeneity of triple-negative breast cancer. *Curr Breast Cancer Rep* 2014; **6**: 154-158.

3 Kumar P, Aggarwal R, An overview of triple-negative breast cancer. *Arch Gynecol Obstet* 2016; **293**: 247-269.

4 Abdel-Fatah TMA, McArdle SEB, Agarwal D, *et al.* HAGE in triple-negative breast cancer is a novel prognostic, predictive, and actionable biomarker: A transcriptomic and protein expression analysis. *Clin Cancer Res* 2016; **22**: 905-914.

5 Rosalia RA, Quakkelaar ED, Redeker A, *et al.* Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. *Eur J Immunol* 2013; **43**: 2554-2565.

6 Bijker MS, van den Eeden SJ, Franken KL, Melief CJ, Offringa R, van der Burg SH, $CD8^+$ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J Immunol*. 2007; **179**: 5033-5040.

7 Speiser DE, Liénard D, Rufer N, *et al.* Rapid and strong human $CD8^+$ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* 2005; **115**: 739-746.

8 Pol J, Bloy N, Buque A, *et al.* Trial watch: peptide-based anticancer vaccines. *Oncoimmunology* 2015; **4**: e974411.

9 Tan AC, Goubier A, Kohrt HE, A quantitative analysis of therapeutic cancer vaccines in phase 2 or phase 3 trial. *J Immunother Cancer* 2015; **3**: 48-60.

10 Metheringham RL, Pudney VA, Gunn B, Towey M, Spendlove I, Durrant LG, Antibodies designed as effective cancer vaccines. *MAbs* 2009; **1**: 71-85.

11 Pudney VA, Metheringham RL, Gunn B, Spendlove I, Ramage JM, Durrant LG, DNA vaccination with T-cell epitopes encoded within Ab molecules induces high-avidity anti-tumor $CD8^+$ T cells. *Eur J Immunol* 2010; **40**: 899-910.

12 Patel PM, Durrant LG, Ottensmeier C *et al.* Phase I trial of ImmunoBody in melanoma patients. *J Clin Oncology* 2014; **32**: 3061-3061.

13 Patel PM, Ottensmeier CH, Mulatero C Targeting gp100 and TRP-2 with a DNA vaccine: Incorporating T cell epitopes with a human IgG1 antibody induces potent T cell responses that are associated with favourable clinical outcome in a phase I/II trial. *Oncoimmunol* 2018**; 7**: e1433516.

14 Herrero-Vicent C, Guerrero A, Gavilá J, *et al.* Predictive and prognostic impact of tumour-infiltrating lymphocytes in triple-negative breast cancer treated with neoadjuvant chemotherapy. *E Cancer Med Science* 2017; **11**: 759-768.

15 Yeong J, Lim J, Lee B *et al.* Prognostic value of $CD8^+$ $PD\text{-}1^+$ immune infiltrates and PDCD1 gene expression in triple-negative breast cancer. *J Immunotherapy Cancer* 2019; **7:** 34-47.

16 Adams S, Diamond JR, Hamilton E *et al.* Atezolizumab plus nab-Paclitaxel in the treatment of metastatic triple-negative breast cancer with 2-year survival follow-up: a Phase 1b clinical trial. *JAMA Oncology* 2019; **5**: 334-342.

17 Mittendorf EA, Philips AV, Meric-Bernstam F, *et al.* PD-L1 expression in triple-negative breast cancer. *Cancer Immunol Res*. 2014; **2**: 361-370.

18 Melief CJ, van der Burg SH, Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat Rev Cancer* 2008; **8**: 351-360.

19 Hailemichael Y, Dai Z, Jaffarzad N, *et al.* Persistent antigen at vaccination sites induces tumor-specific $CD8^+$ T cell sequestration, dysfunction and deletion. *Nat Med* 2013; **19**: 465-472.

20 Chiang CL-L, Kandalaft LE, Coukos G, Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. *Int Rev Immunol* 2011; **30**: 150-182.

21 Khong H, Overwijk WW, Adjuvants for peptide-based cancer vaccines. *J Immunother Cancer* 2016; **4**: 56-67

22 Haining WNW, Davies J, Kanzler H, *et al.* CpG Oligodeoxynucleotides Alter Lymphocyte and Dendritic Cell Trafficking in Humans. *Clin Cancer Res* 2008; **14**: 5626-5634.

23 Valmori D, Souleimanian NE, Tosello V, *et al.* Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci USA* 2007; **104**: 8947-8952.

24 Baumgaertner P, Jandus C, Rivals JP, *et al.* Vaccination-induced functional competence of circulating human tumor-specific CD8 T-cells. *Int J Cancer* 2012; **130**: 2607-2617.

25 Almshayakhchi R, Nagarajan D, Vadakekolathu J, *et al*. A novel HAGE/WT1-ImmunoBody® vaccines combination to treat leukaemia. *Frontiers in Oncology* 2021 doi: 10.3389/fonc.2021.636977

26 Martelange V, De Smet C, De Plaen E, *et al*. Identification on a human sarcoma of two new genes with tumor-specific expression. *Cancer Res* 2000; **60**: 3848-3855.

27 Nagel H, Laskawi R, Eiffert H, *et al*. Analysis of the tumour suppressor genes, FHIT and WT-1, and the tumour rejection genes, BAGE, GAGE-1/2, HAGE, MAGE-1, and MAGE-3, in benign and malignant neoplasms of the salivary glands. *Mol Pathol* 2003; **56**: 226-231.

28 Linley AJ, Mathieu MG, Miles AK, Rees RC, McArdle SEB, Regad T The helicase HAGE expressed by malignant melanoma-initiating cells is required for tumor cell proliferation *in vivo*. *J Biol Chem* 2012; **287**: 13633-13643.

29 Mathieu MG, Knights AJ, Pawelec G, *et al.* HAGE, a cancer/testis antigen with potential for melanoma immunotherapy: identification of several MHC class I/II HAGE-derived immunogenic peptides. *Cancer Immunol Immunother* 2007; **56**: 1885-1895.

30 Xue W, Brentville VA, Symonds P, *et al.* SCIB1, a huIgG1 antibody DNA vaccination, combined with PD-1 blockade induced efficient therapy of poorly immunogenic tumors. *Oncotarget* 2016; **7**: 83088-83100.

31 Xue W, Metheringham RL, Brentville VA, *et al.* SCIB2, an antibody DNA vaccine encoding NY-ESO-1 epitopes, induces potent antitumor immunity which is further enhanced by checkpoint blockade. *Oncoimmunol* 2016; **5**: e1169353.

32 Wada S, Jackson CM, Yoshimura K, *et al.* Sequencing CTLA-4 blockade with cell-based immunotherapy for prostate cancer. *J Transl Med* 2013; **11**: 89-103.

33 Remy-Ziller C, Thioudellet C, Hortelano J, *et al.* Sequential administration of MVA-based vaccines and PD-1/PD-L1-blocking antibodies confers measurable benefits on tumor growth and survival: Preclinical studies with MVA-βGal and MVA-MUC1 (TG4010) in a murine tumor model. *Hum Vaccin Immunother* 2013; **14**: 140-145.

34 McNeel DG, Eickhoff JC, Wargowski E, *et al.* Concurrent, but not sequential, PD-1 blockade with a DNA vaccine elicits anti-tumor responses in patients with metastatic, castration-resistant prostate cancer. *Oncotarget* 2018; **9**: 25586-25596.

Table 1. Peptides derived from HAGE 30mer QTGTGKTLCYLMPGFIHLVLQPSLKGQRNR.

Peptide number	Peptide position	Sequence	Syfpeithi score	length	HLA-Haplotype
P3	287-296	QTGTGKTLCY	25	10mer	HLA-A1
P4	297-305	LMPGFIHLV	28	9mer	HLA-A2
P5	296-304	YLMPGFIHL	27	9mer	"
P6	296-305	YLMPGFIHLV	30	10mer	"
-	303-311	HLVLQPSLK	25	9mer	HLA-A3
P7	291-305	GKTLCYLMPGFIHLV	30	15mer	HLA-DR1
P8	298-312	MPGFIHLVLQPSLKG	26	15mer	"
-	305-309	CYLMPGFIHLVLQPS	26	15mer	HLA-DR4
-	309-313	PGFIHLVLQPSLKGQ	26	15mer	"

Figure 1. *Ex vivo* IFNγ ELISpot assay using splenocytes from mice immunised with HAGE 30mer peptide vaccine with different adjuvants. IFN-γ cytokine release byIFNγ ELISPOT assay using freshly isolated splenocytes derived from mice immunized with HAGE 30mer peptide formulation containing IFA, CpG and IFA+CpG. Graph shows number of IFNγ spots released by splenocytes upon stimulations with MHC Class I (1 μg mL^{-1}) and Class II peptide at 1 μg (10 μg mL^{-1} and 10 μg mL-final concentration respectively at 37°C to measure the immune response induced by) individual HAGE-derived short peptides. Data show that IFA with CpG in combination induces significantly higher number of IFN-γ releasing cells compared to cells induced by IFA or CpG individually. All data presented are represented as means ± SEM (n = 3, 3 mice per group in each independent experiment).

Figure 2. *Ex vivo ELISpot assay:* Comparison between the ImmunoBody®-HAGE vaccine and 30mer peptide vaccine (IFA+CpG). IFN-γ cytokine release by **(a)** Interferon-gamma ELISPOT assay using freshly isolated splenocytes derived from mice immunized with HAGE-ImmunoBody® or HAGE-30mer peptide + IFA/CpG or no immunization plated with MHC Class I (1μg mL^{-1}) and Class II peptide at 1 μg mL^{-1} and 10 μg mL^{-1} final concentration respectively at 37°C to measure the immune response induced by individual HAGE-derived short peptides between different immunisation groups compared to naïve. All data presented (10 μg mL^{-1}) peptide. Functional avidity of **(b)** peptide 5 and **(c)** peptide 6. All data are represented as means ± SEM (n = 3, 3 mice in each independent experiment).

Figure 3. Establishment of stable B16 cell line (knockout for murine β2-microglobulin and then transfected with chimeric HHDII and HLA-DR1) to co-express HAGE and Luciferase.
B16 cells transduced with lentiviral particles from vector with and without HAGE were analysed for HAGE expression. **(a)** HAGE mRNA levels in B16 transfectants showing 3 technical replicates. **(b)** Representative western blotting for HAGE expressions. The bands shown are from the same blot but probed with HAGE protein on top and B actin at the bottom, with PCI13 cell lysate used as a positive control for HAGE protein. **(c)** Immunofluorescence staining of B16/HHDII/DR1 cells transfected with empty vector and HAGE. Cells were stained with DAPI (blue, nuclear staining) and primary DDX43antibody (red) shown at magnification x4, **(d)** flow cytometry analysis of HLA-DR1 and B2M expression, **(e)** luciferase expression measured as luminescence (total flux) with D-luciferin (15 μg mL^{-1}) expressed activity in B16/HAGE, Luc2 cells. HAGE expression in TNBC cells **(f)** mRNA and **(g)** protein levels.

Figure 4. HAGE specific $CD8^+$ T cell responses after *in vitro* stimulation: ELISpot assay. (a) HAGE-specific target induced IFNγ cytokine release by pure $CD8^+$ T cells after 48 h of co-culture with un-pulsed and peptide-pulsed (100 μg mL^{-1}) T2 cells, B16 +/- HAGE, human TNBCs (MDA-MB-231, MDA-MB-468) cell lines for 48 h to measure the IFN-γ induced due to presence of target cells. HAGE-specific target induced IFN-γ cytokine release was observed following incubation with HLA-$A2^+$, $HAGE^+$ cell lines. Splenocytes from immunised mice were co-cultured *in vitro* with LPS activated cells and plated with ^{51}Chromium labelled-target cells. ^{51}Chromium release assay to determine HAGE specific cytotoxicity against **(b)** T2 cells pulsed overnight with cocktail of MHC Class I peptides, **(c)** B16-/+HAGE cells, **(d)**

ImmunoBody®-HAGE vaccine-induced T cell cytotoxicity against MDA-MB-231 and MDA-MB-468 cells. Groups were compared to obtain statistical P values obtained by two-way ANOVA analysis. All data presented are mean ± SEM, (n = 2, 3 mice/group) tested in triplicates in each independent experiments.

Figure 5. Efficacy of ImmunoBody®-HAGE vaccine against B16/HHDII/DR1/HAGE/Luc subcutaneous tumours. Tumour bearing animals were monitored for tumour growth by measuring luciferase activity. **(a)** Representative images of tumour bearing mice observed over time, **(b, c and d)** comparison of total flux between control and treatment groups and **(e)** Survival curves showing growth of subcutaneous tumours (n = 1, 11 mice per group). One experiment. 3 representative images are shown for each group.

Figure 6. Efficacy of ImmunoBody®-HAGE vaccine in a metastasis model. Mice were intravenously injected with B16/HHDII/DR1/HAGE/Luc cells. **(a)** Representative images of tumour bearing mice observed over time, **(b)** comparison of total flux between control and treatment groups and **(c)** Survival curves indicating the proportions of surviving mice in each group (n = 1, 10 per group). Survival curve analysis was performed using the Gehan-Breslow-Wilcoxon test to obtain significant differences (*P*-value) between control and treatment groups. One experiment. 3 representative images are shown for each group

Figure 7. Additional vaccinations after tumour implantation on pre-vaccinated animals significantly increase time to death, which was not further improved by the injection of anti-PD1 antibody.
Mice (n = 10 per group) were all vaccinated 3 times (once per week) with ImmunoBody®-HAGE, thereafter all animals received B16/HHDII$^+$/DR1$^+$/HAGE$^+$ cells and a further 3 injections of the vaccine with/without the addition of anti-PD1. Results demonstrate the benefit of further vaccination after the tumour injection with a significant delay before mice had to be culled due to tumour size. The addition of anti-PD-1 did not, however, further improve this outcome.

Figure 8. M2-type macrophages are significantly increased in large tumours. TILs from all tumours (with and without anti-PD1) were extracted and phenotypic analysis was performed using flow cytometry. Results demonstrate that while no differences could be found in the proportion of macrophages, the proportion of M2 macrophages (CD206$^+$/IL-10$^+$) was significantly increased in larger tumours.

Figure 9. Large tumours contain proportionally more regulatory T-cells than smaller ones, but no more *MDSCs*. TILs from small, large and tumour only groups were assessed by flow cytometry. Although no significant difference could be found in the proportion of CD3$^+$CD4$^+$, the percentage of CD25$^+$FoxP3$^+$ Treg cells within this population was significantly higher in tumours with the largest weight. No differences were found in the MDSC compartments.

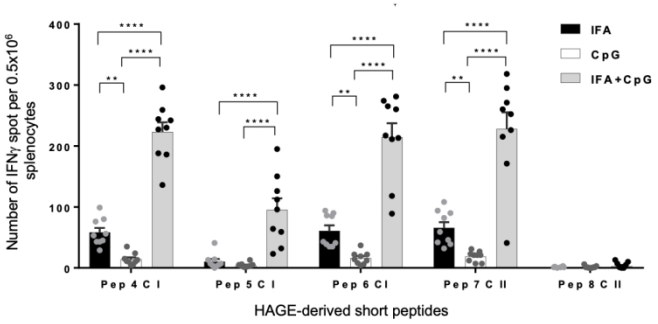
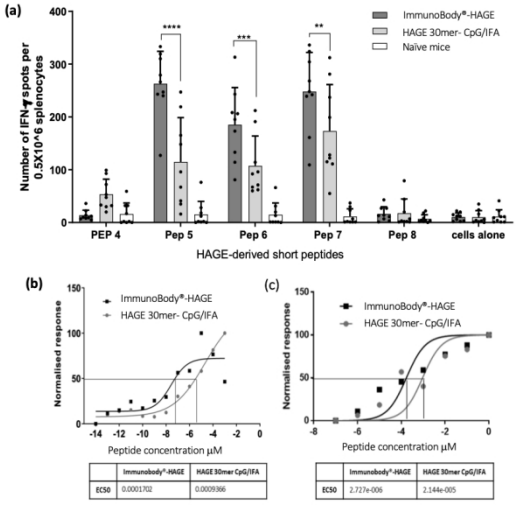


Figure 1

imcb_12485_f1.jpg



	ImmunoBody®-HAGE	HAGE 30mer CpG/IFA
EC50	0.0001702	0.0009366

	ImmunoBody®-HAGE	HAGE 30mer CpG/IFA
EC50	2.727e-006	2.144e-005

Figure 2

imcb_12485_f2.jpg

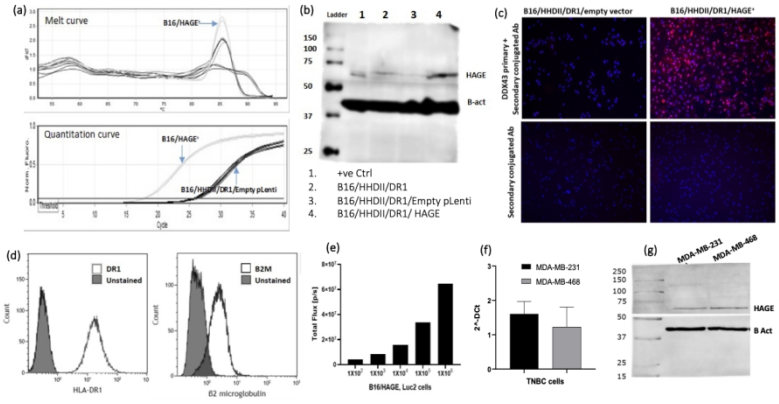


Figure 3

imcb_12485_f3.jpg

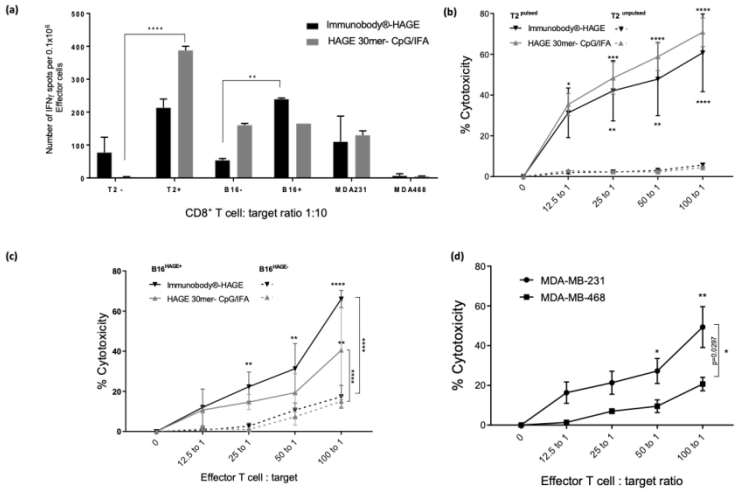


Figure 4

imcb_12485_f4.jpg

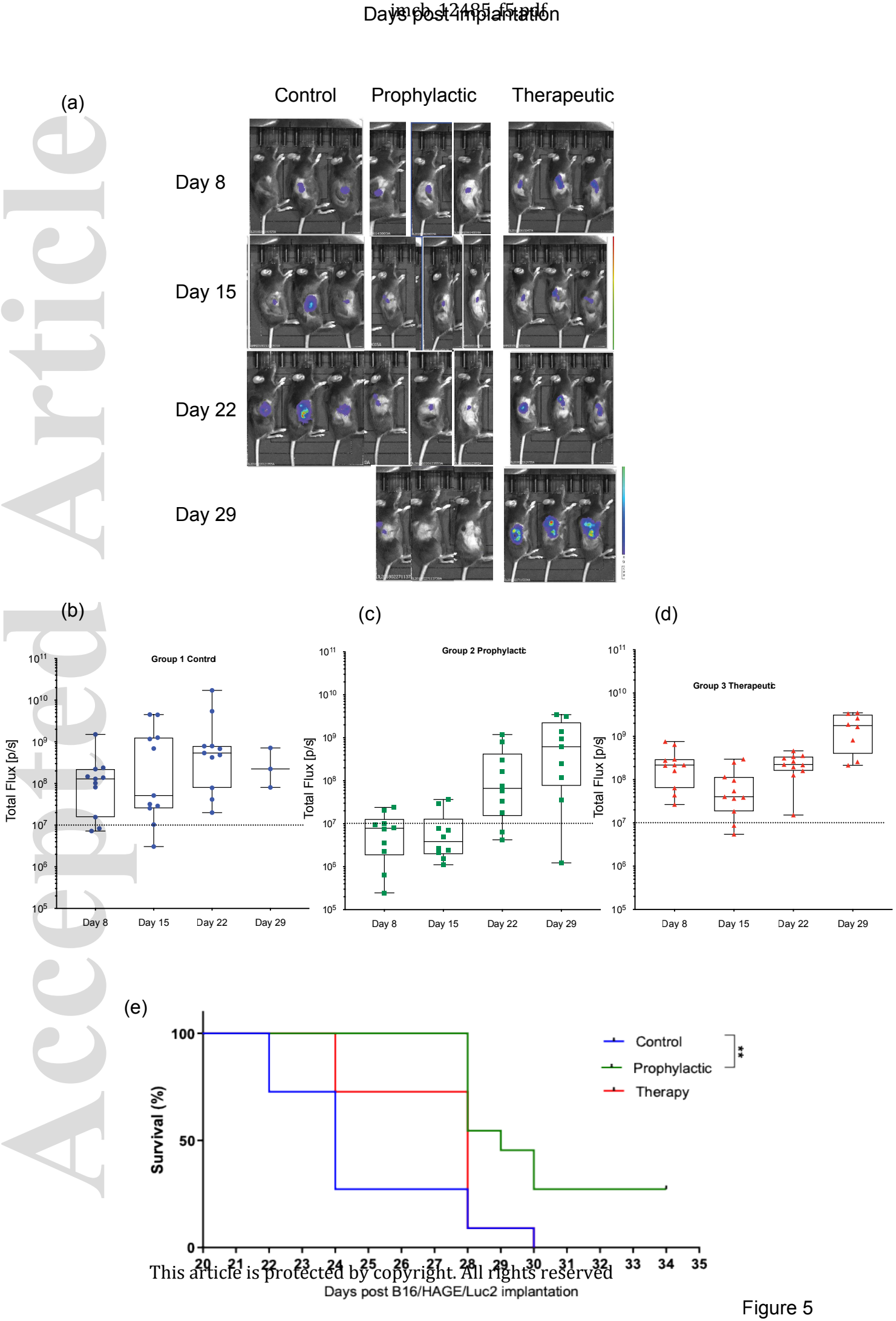
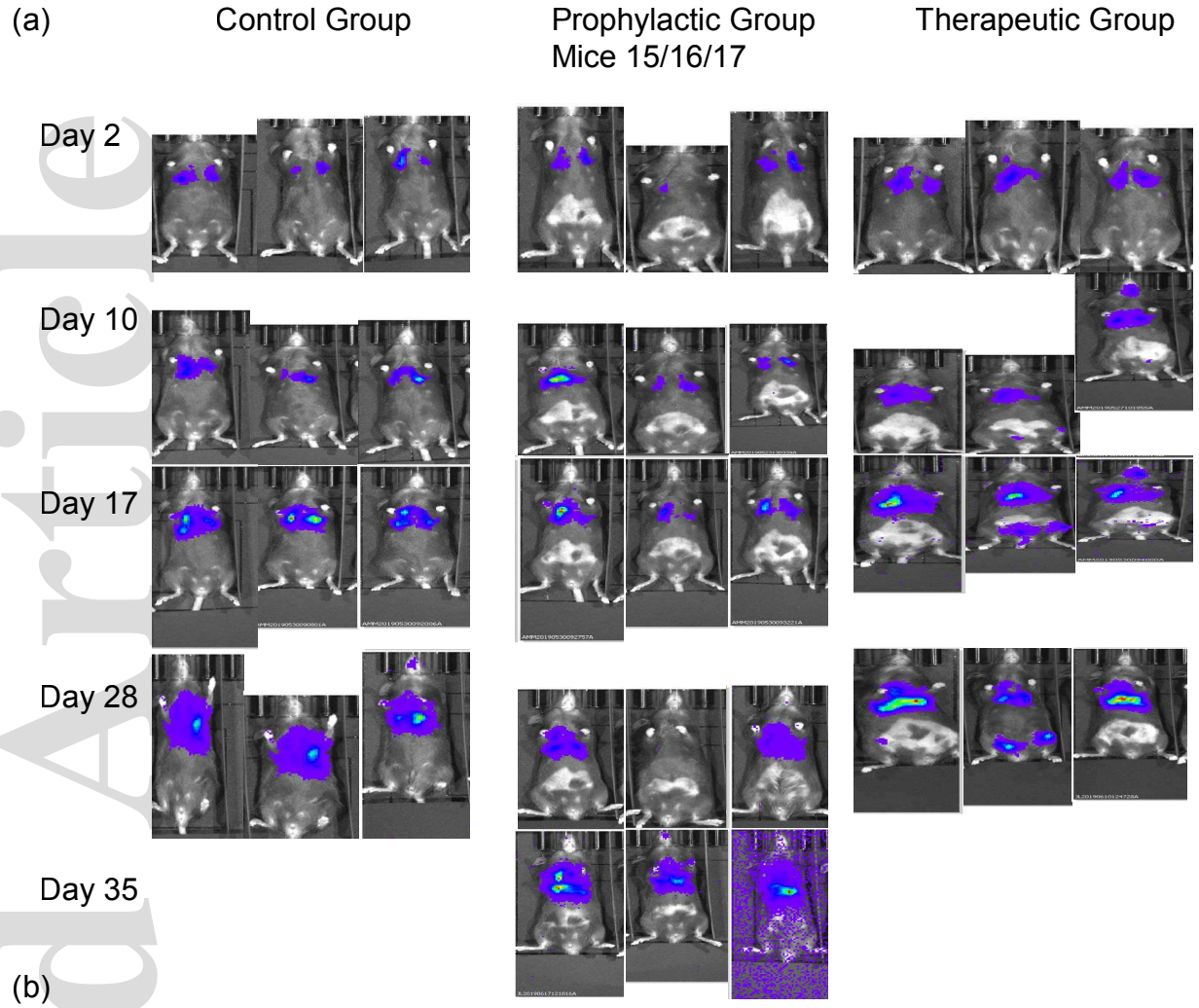
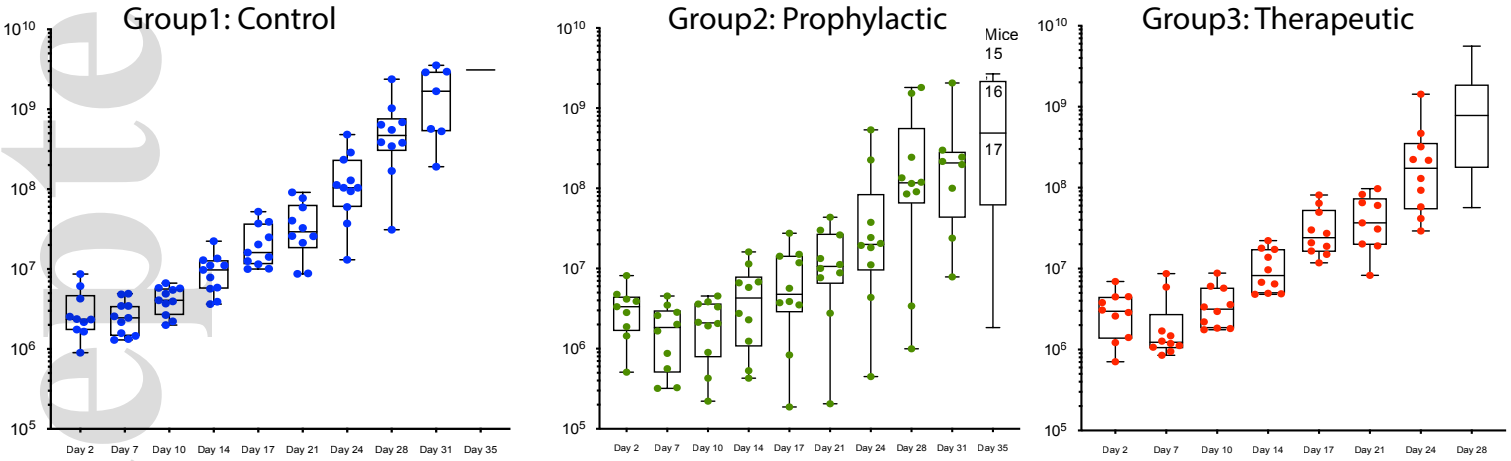


Figure 5





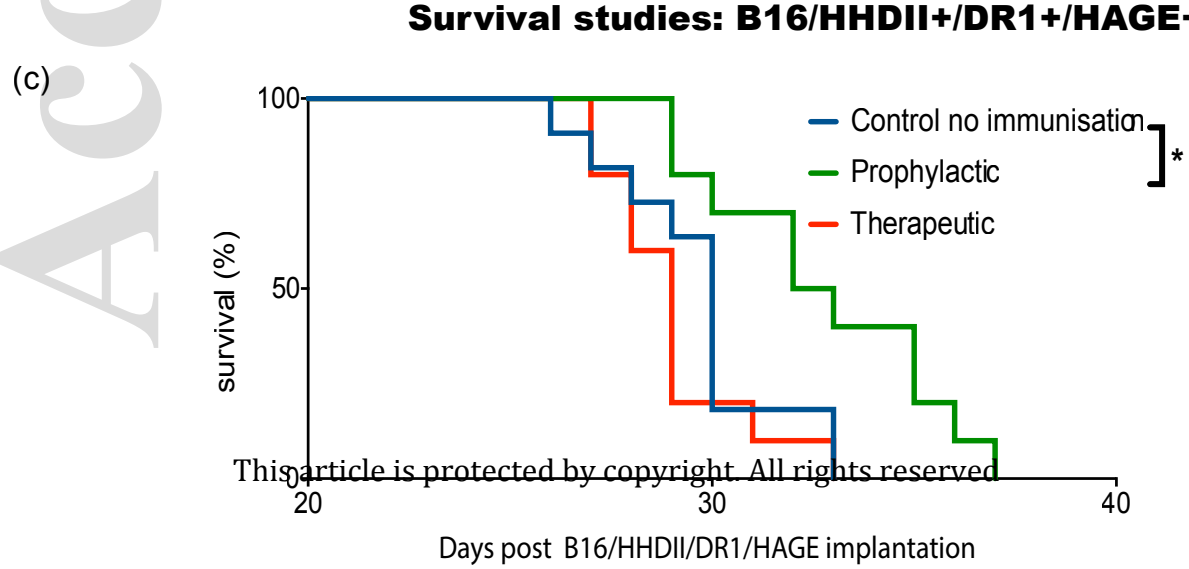


Figure 6

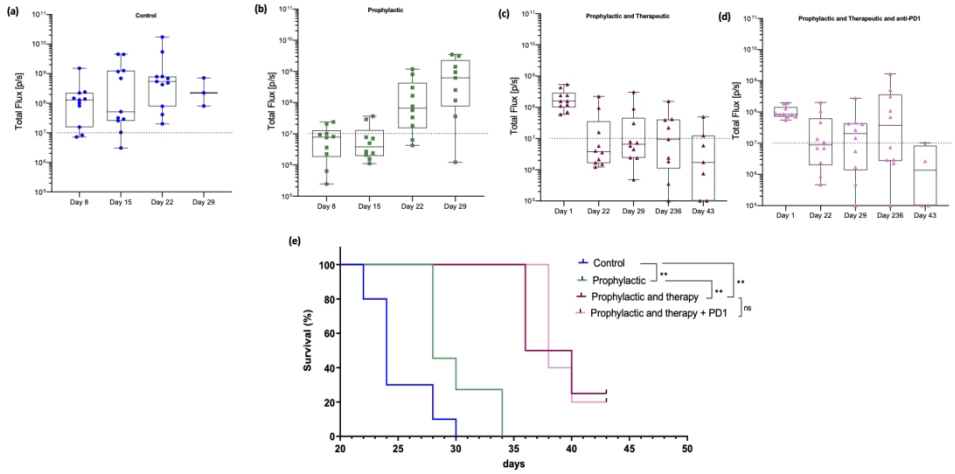


Figure 7

imcb_12485_f7.jpg

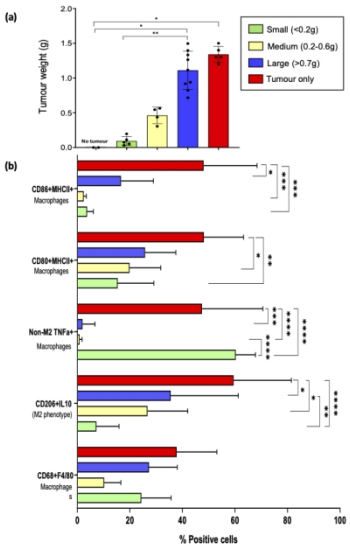


Figure 8

imcb_12485_f8.jpg

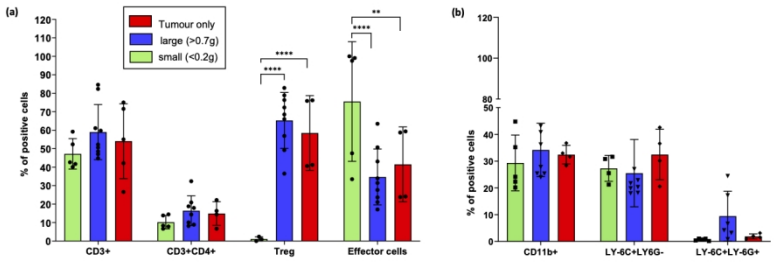


Figure 9

imcb_12485_f9.jpg