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Targeting DNGR-1 (CLEC9A) with antibody/MUC1 peptide conjugates as a vaccine for carcinomas

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DCs are the most potent APCs and are the focus of many immunotherapeutic approaches for the treatment of cancer, although most of these approaches require the ex vivo generation and pulsing of DCs. We have targeted a subset of DCs in vivo using an Ab to DNGR-1, a C-type lectin dedicated to the cross-presentation of Ag expressed by subsets of DCs. HLA-A2 epitopes from the tumour-associated Ag, MUC1, were coupled to the anti-DNGR-1 Ab, and their efficacy in generating a Th1-cell response and inhibiting tumour growth was evaluated in a clinically relevant double transgenic mouse model expressing human MUC1 and A2K/b. Using this strategy, we demonstrate that an effective immune response to MUC1 can be generated, which results in a significant delay in the growth of MUC1-expressing tumours in both prophylactic and therapeutic settings. In addition, we also show, using PBMCs isolated from healthy volunteer blood, that target an MUC1 HLA-A2 epitope to human DNGR-1 in vitro can induce an MUC1-specific $CD8^+$-T-cell response, which confirms the relevance of our in vivo murine results in the human setting.

Keywords: Cancer · Cross-presentation · Dendritic cells · Immunotherapy

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Introduction

Active immunotherapy aimed at stimulating the patient's immune response has, on the whole, resulted in disappointing results in the clinic [1]. Immunisation with protein Ags often only elicits $CD4^+$ T-cell responses without the induction of $CD8^+$-T-cell responses, as exogenous protein is primarily presented to $CD4^+$ T cells [2]. DCs are the most potent APCs having the ability to orchestrate a repertoire of immune responses, and thus are the key players in the initiation and control of the innate and adaptive immune response. As such, considerable interest has been focussed on directing antigen to these cells for the immunotherapy of cancer. Since 2008, there have been around 100 clinical trials based on DCs and all but one involve the ex vivo pulsing of DCs differentiated in vitro from monocytes or haematopoietic stem cells [3]. However, only one study has progressed beyond the phase I or II stage, as further clinical developments are challenging. The one exception is Sipuleucel-T (Provenge) that was approved by the FDA in 2010 for use in patients with asymptomatic or minimally symptomatic metastatic hormone-refractory prostate cancer [4]. However, in this protocol, autologous monocytes are harvested and pulsed ex vivo with a fusion protein of prostatic acid phosphatase and GM-CSF, and the pulsed monocytes are returned to the patient for maturation in vivo [5].

The high labour intensity and cost of ex vivo pulsing, together with uncertainties concerning the trafficking of ex vivo differentiated DCs and their efficacy of cross-presentation, has led to strategies to target DCs with Ag in vivo. C-type lectins are found on APCs such as macrophages and DCs, and although their ligands are usually carbohydrate, they can include protein and lipids [6].

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Binding of ligands to C-type lectins triggers Ag uptake and secretion of cytokines such as interferons and interleukins, allowing this arm of the innate immune system to act as a first line of defence against pathogens [7]. However, Ags internalised through these C-type lectins can also be processed for presentation to T cells [7].

Targeting lectins on DCs has been increasingly used in preclinical models for the in vivo targeting of Ags to these cells [8]. This has usually been achieved by using Abs to C-type lectins expressed by DCs but the use of their natural ligands is also being explored [9, 10]. C-type lectins DEC-205, DCIR-2, DC-SIGN, MGL and the mannose receptor have been used to target a number of different Ags to DCs [9, 11–14]. However, DCs consist of a number of phenotypically distinct subsets, which differ in their function, with some being better at cross-presentation than others [15]. Mouse CD8α$^+$ DCs, which are characterised by the expression of the transcription factor BATF3 [16], are extremely efficient at cross-presentation of Ag on MHC class I, whereas CD8α$^-$ DCs are less effective. Indeed, targeting CD8α$^-$ DCs has been shown to be more efficient at inducing CD4$^+$ T-cell responses, while targeting CD8α$^+$ T cells induces CD8$^+$ T-cell responses [17].

CD8α$^+$ DCs can capture material from dead or dying cells, which can be cross-presented on MHC class I [18–20]. These functions are executed by the C-type lectin DNGR-1 (also called CLEC9A), which is dedicated to the cross-presentation of dead cell associated Ags leading to the induction of cytotoxic T lymphocytes (CTLs). It has been shown that targeting Ag to DNGR-1 via coupling ovalbumin (OVA) or endogenous B16 melanoma peptides to antibodies to DNGR-1, can result in the decrease of pseudometastases in an OVA model of the B16 mouse melanoma tumour [21]. DNGR-1 is restricted in its expression, being found on the CD8α$^+$ and CD103$^+$ CD11b$^-$ subsets of DCs [16]. Importantly, cells bearing a similar phenotype, which are BATF3-dependent and express DNGR-1, have now been identified in the human DC network [16, 22].

MUC1 is over-expressed by the majority of adenocarcinomas, and in a study to prioritise cancer Ags, it was ranked the second most optimal Ag for vaccine development [23]. Several clinical studies have used DCs loaded ex vivo with MUC1 peptides in a range of cancer patients, and over a score of small trials have attempted a vaccine approach injecting an MUC1 peptide, often coupled to keyhole limpet hemocyanin and given with adjuvant. Most of these studies have focussed on using tandem repeat based peptides [24], which do not contain high affinity class I peptides, with very few using peptides outside the tandem repeat [25]. However, CD8$^+$ T-cell recognising MUC1 epitopes have been demonstrated in the blood of cancer patients, but these are reactive with non-tandem repeat based peptides [26, 27].

Preclinical testing of immune-based therapies is often hindered by the model systems available. Here, we use a double transgenic mouse model expressing human MUC1 and A2K/b to evaluate human MHC class I epitopes for their efficacy in the immunotherapy of MUC1-expressing tumours. These mice are on a pure C57BL/6 background and express human MUC1 under its own promoter [28] and A2K/b that is a modified class I MHC molecule combining the alpha-1 and alpha-2 domains of human HLA-A*0201 with the alpha-3 domain of the mouse H-2Kb [29]. We demonstrate that targeting HLA-A2 epitopes from MUC1 to DNGR-1 can cause a delay in tumour growth in a prophylactic and therapeutic setting. In addition, we also show, using PBMCs isolated from healthy volunteer blood, that targeting an MUC1 HLA-A2 epitope to human DNGR-1 in vitro can induce a CD8$^+$ T-cell response to both peptide-pulsed targets and MUC1 positive, HLA-A2 tumour cell lines, thus confirming the relevance of our in vivo murine results to the human setting. Taken together, our results show that targeting DNGR-1 with MUC1 class I peptides is an attractive immunotherapeutic strategy.

Results

HLA-A2 epitopes within MUC1 generate a T-cell response in MUC1xA2K/b mice

HLA-A2 class I peptides ALGSTPPV (ALG), FLSFHISNL (FLS) and LLLLTVLTV (LLLL), previously identified in MUC1 and shown to be endogenously processed [25, 30], were coupled to anti-mouse DNGR-1 as described in the *Materials and methods*. This coupling resulted in a ratio of 0.5, 2.5 and 0.25, respectively, of peptide molecules coupled per molecule of Ab. Various ratios of peptide and Ab were tried in the coupling procedure (data not shown) with a 5:1 ratio of peptide to Ab giving the results reported here. CD11c$^+$DNGR-1$^+$ cells were shown to be present in MUC1 transgenic mouse spleens, with MUC1 being virtually undetectable on these cells (Supporting Information Fig. 1). This is in contrast to the high expression of MUC1 seen by the MC38 cells as shown in Figure 2A. MUC1xA2K/b transgenic mice were vaccinated with the peptides coupled to DNGR-1 or isotype control together with anti-CD40 and polyinosinic:polycytidylic acid (poly-IC), and 7 days later splenocytes from the vaccinated mice were stimulated in vitro with the individual peptides. As shown in Figure 1A, peptides ALG and LLLL induced the secretion of IFN-γ in some of the mice vaccinated after only one injection of the self-immunogen coupled to DNGR-1; one of four for ALG and two of four for LLLL. Experiments to determine immunogen concentration and dosing schedule demonstrated that 1 or 2 μg of Ab coupled with peptide gave an equivalent response (data not shown) but three vaccinations 2 weeks apart induced the strongest and most consistent response with four of five mice responding (Fig. 1B). No response was observed with the peptides coupled to an isotype control Ab demonstrating the involvement of DNGR-1 in orchestrating the response.

Targeting MUC1 class I peptides to DNGR-1 inhibits tumour growth

To determine if the induced immune response to MUC1 can inhibit tumour growth in a prophylactic model, MUC1xA2K/b transgenic mice were vaccinated with three injections of the ALG and LLLL peptides coupled to anti-DNGR-1 or isotype control, or PBS,

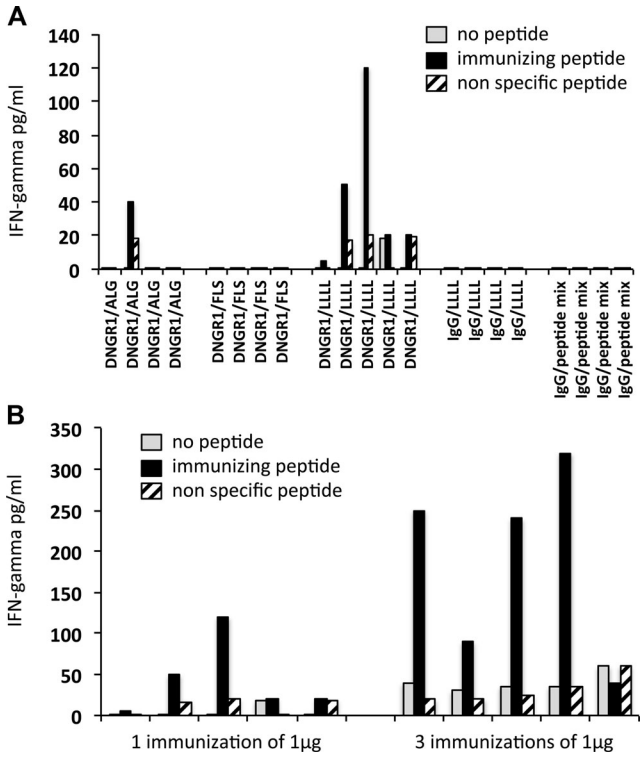

Figure 1. Immunisation of MUC1xA2K/b transgenic mice with MUC1 HLA-A2 peptides coupled to anti-DNGR-1 induces an immune response. (A) MUC1xA2K/b transgenic mice were vaccinated with a single injection of the peptides coupled to DNGR-1 or isotype control together with anti-CD40 and poly-IC. Seven days later, splenocytes from the vaccinated mice were stimulated in vitro with the individual peptides and the supernatant assayed for IFN-γ production by ELISA in triplicate. Results shown are for individual mice, either four or five per group as indicated. (B) MUC1xA2K/b transgenic mice were vaccinated with either a single or three injection(s) (2 weeks apart) of LLLL MUC1 peptide coupled to DNGR-1 with anti-CD40 and poly-IC (five mice per group per time point). Seven days following the final injections, splenocytes from the vaccinated mice were stimulated in vitro with the LLLL or another MUC1 peptide (non-specific) and IFN-γ production evaluated by ELISA, each point being assayed in triplicate. Results from individual mice are shown.

together with anti-CD40 and poly-IC. One week following the final injection, mice were challenged with the MC38 murine tumour transfected with MUC1 and A2K/b (MC38-MUC1-A2; Fig. 2A). Figure 2B and C shows that vaccination with the MUC1 peptides coupled to anti-DNGR-1 significantly reduced tumour growth compared with tumour growth after vaccination with isotype control coupled peptides (Fig. 2B, $p = 0.0253$) or vehicle alone. The experiment was repeated and the combined results of the two independent experiments are shown in Figure 2D, where a significant difference in tumour growth is observed between mice vaccinated with MUC1 peptides coupled to anti-DNGR-1 and the controls ($p = 0.0336$).

To determine if targeting DCs with MUC1 HLA-A2 peptides via DNGR-1 resulted in reduced tumour growth in the therapeutic setting, MUC1xA2K/b transgenic mice were given MC38-MUC1-A2 tumour cells, and 4 and 14 days later, the mice were injected with peptides coupled to anti-DNGR-1 or to the isotype control in the presence of anti-CD40 and poly-IC (Fig. 3A). Figure 3B shows the tumour-free survival of the mice and Figure 3C the growth of tumours in the individual mice. These data show that giving the anti-DNGR-1 vaccine once the tumour had been established also resulted in a significant decrease in the growth of the tumours compared with that in the isotype control (Fig. 3B, $p = 0.0143$). The experiment was repeated and Figure 3D shows the combined results of two independent experiments and demonstrates that treating MUC1xA2K/b transgenic mice with MUC1 HLA-A2 peptides coupled to an anti-DNGR-1 Ab reproducibly resulted in a significant reduction in tumour growth ($p = 0.0021$). The results are particularly encouraging, as for the LLLL and ALG peptides used in these in vivo tumour experiments we could only obtain coupling ratios of 0.25 and 0.5 molecules of peptide to Ab, respectively, indicating the presence of non-conjugated Ab that may compete to some degree for binding.

Targeting the LLLTVLTV peptide to human DNGR-1 can induce CD8$^+$ T-cell response in vitro

It has recently been established that a population of DCs equivalent to mouse CD8α can be found in humans [22] and characterised as being BDCA-3^{hi}, DNGR-1$^+$. However, these cells are present in very small numbers in peripheral blood. Given this caveat, we investigated if targeting human DNGR-1 with MUC1 peptides could stimulate naïve T cells to recognise MUC1. BDCA-3 positive cells were isolated from PBMCs and incubated with the LLLTVLTV (LLL) peptide or isotype control, coupled to anti-human DNGR-1 (peptide Ab coupling ratio of 0.28 and 0.20, respectively) in the presence of anti-CD40 and poly-IC, for 1 hour before incubating with autologous T cells for 7 days. After 7 days, the cultures were stimulated with peptide, incubated for another 7 days before re-stimulating with peptide-pulsed autologous monocyte-derived DCs. IFN-γ production by CD8$^+$ T cells in response to peptide or MUC1-expressing human HLA-A2 tumour cell lines was determined 7 days later. Figure 4A shows a representative plot of four independent experiments using two different HLA-A2$^+$ donors and illustrates that an initial stimulation with LLL coupled to anti-DNGR-1 could specifically stimulate a small but consistent population of CD8$^+$ T cells that could respond to further stimulation with MUC1 peptide by the secretion of IFN-γ. Importantly, MUC1 peptide coupled to DNGR-1 induced T cells that recognised HLA-A2 human tumour cells (MCF-7 and Capan-1) expressing endogenously processed MUC1 (Fig. 4B and C) responding by the production of IFN-γ. This was in contrast to that observed when the initial stimulation was with the same peptide coupled to the isotype control (Fig. 4B and C, Ig/LLL panels).

Discussion

DCs are key players in stimulating the immune response, and as such they have become an important focus for immunotherapeutic strategies. The vast majority of clinical studies have focussed on the ex vivo pulsing of DCs derived in vitro but this approach has

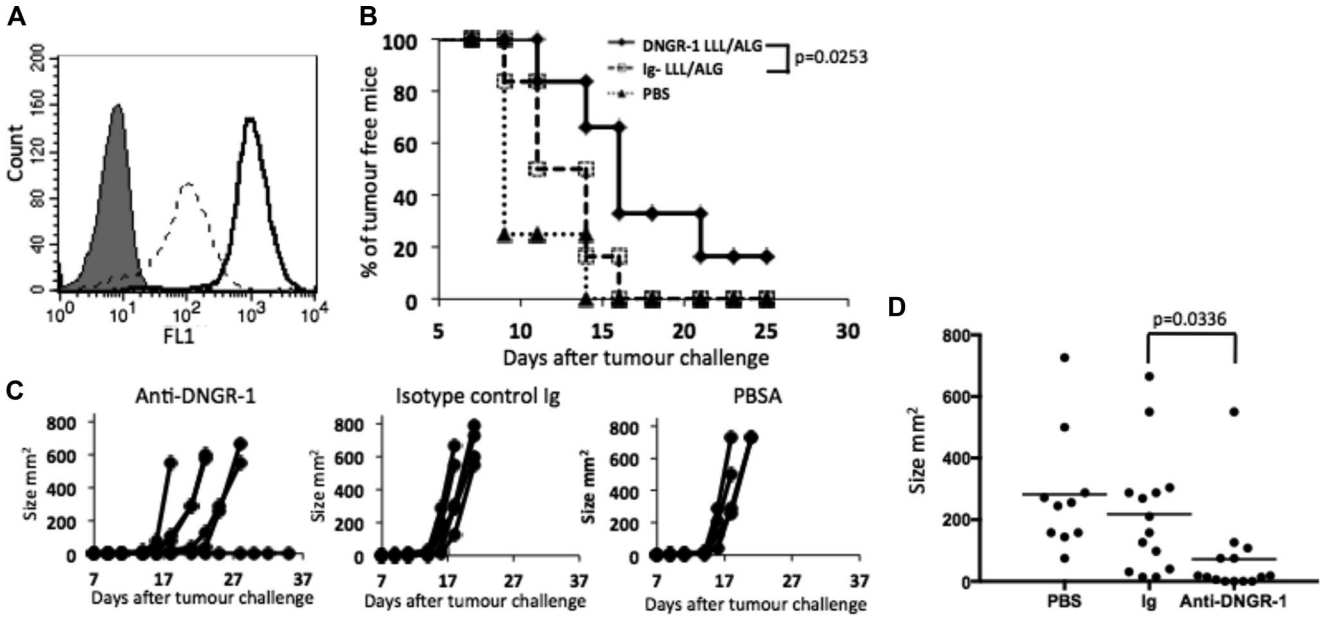

Figure 2. Immunisation of MUC1xA2K/b transgenic mice with MUC1 peptides coupled to anti-DNGR-1 inhibits the growth of MUC1-A2K/b-expressing tumours. (A) Flow cytometric analysis of MC38 murine tumour cells expressing human MUC1 and A2K/b using the mAbs HMFG2 (MUC1, solid line histogram), BB7.2 (HLA-A2, dotted line histogram) or isotype control (filled histogram). (B–D) MUC1x A2K/b transgenic mice were vaccinated with three injections of the ALG and LLLL peptides coupled to anti-DNGR-1 or isotype control, or PBSA, together with anti-CD40 and poly-IC. One week following the final injection, mice were challenged with the MC38-MUC1-A2K/b. (B) Tumour-free survival was measured for six DNGR-1 mice, six isotype control mice and four PBS mice; statistical analysis between the anti-DNGR-1 and isotype control groups was performed using the Mantel–Cox test. Data are from a single experiment representative of two performed. (C) Growth of the tumours in individual mice was also measured and shown as individual growth curves for single mice (anti-DNGR-1 and isotype control group $n = 6$ mice per group, PBSA $n = 4$). (D) Size of tumours at day 14 is shown, combining the results of two independent experiments. *p* Values were determined using an unpaired *t*-test.

resulted in only limited success and is a complex, time-consuming and expensive procedure. The targeting of Ag in vivo to DCs is a more attractive and viable strategy. Here, we have demonstrated the effective in vivo targeting of HLA-A2 peptides from MUC1 to DCs via an Ab to DNGR-1. This resulted in a reduction of tumour growth in an MUC1 transgenic mouse model. Moreover, the use of MUC1xA2K/b mice makes the results obtained in this highly relevant model directly applicable to humans and clinical trials.

It is important to target Ag to the correct DCs subset in vivo if the desired response is to be obtained. In the mouse, CD8α$^+$ DCs are extremely efficient at cross-presenting Ag on MHC class I for the induction of CTLs. Both DEC-205 and DNGR-1 are expressed on CD8α$^+$ DCs. However, DEC-205 is also expressed on other cell types including other subsets of DCs, B cells, macrophages and T cells and human DEC-205 shows an even broader expression pattern. This will result in only a small proportion of the Ab complex being delivered to the relevant DCs subtype. In contrast, DNGR-1 is more restricted in its expression pattern being confined to the CD8α$^+$ and CD103$^+$ CD11b$^-$ DCs in the mouse and marking a CD11b$^-$ DC population in humans [16]. This makes it an ideal endocytic receptor to target. It has been shown to play a pivotal and non-redundant role in cross-presenting cell-associated Ags from dying cells [18], and viral Ags exposed in viral induced cell death [19, 20].

C-type lectins involved in Ag uptake are more abundantly expressed on immature DCs, which upon activation down-regulate Ag uptake receptors and up-regulate MHC and co-stimulatory molecules [6, 7, 15]. Targeting DCs without co-stimulation is believed to induce tolerance and indeed targeting DEC-205 without DC-activating adjuvants results in tolerance [31], whereas targeting this receptor in the presence of co-stimulators results in the generation of CTLs and Abs. Recent studies have shown that DNGR-1 may act as a 'neutral' receptor in that Ag engagement of this lectin induces cross-presentation but does not activate DCs, helping to promote tolerance to autologous cellular Ags [20, 32]. Targeting DNGR-1 in the absence of adjuvant with small amounts of Ag/Ab complexes can induce tolerance by driving CD4$^+$ T cells to FOXP3$^+$ Treg cells [32]. In contrast, others have reported that targeting DNGR-1 in the absence of adjuvant can induce a humoral response [33]. However, the use and type of adjuvant can direct a Th2 or Th1 response [34]. Importantly, poly-IC was particularly efficient at directing a Th1 response [34]. In the strategy used here, MUC1 HLA-A2 peptides were targeted to DNGR-1 in the presence of the TLR3 ligand poly-IC and anti-CD40. This resulted in an effective Th1 response and the decreased growth of established tumours.

The human equivalent DC subset to the CD8α$^+$ cells found in mice has been, until recently, very elusive. However, a subset of

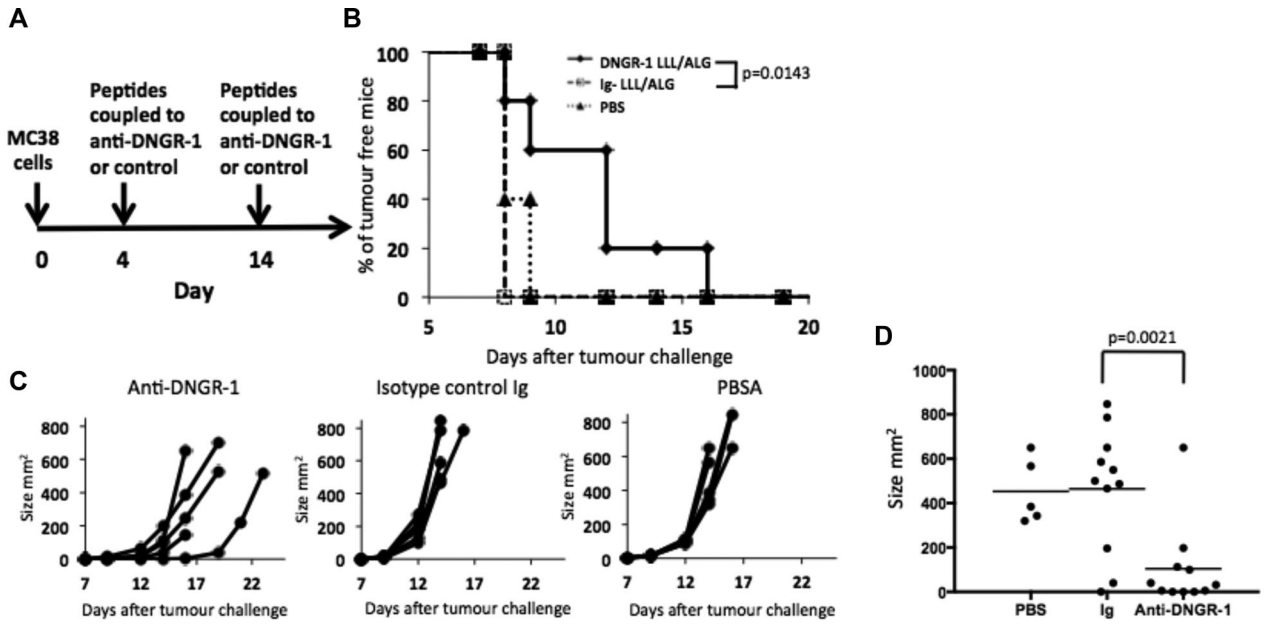

Figure 3. Treatment of MUC1xA2K/b transgenic mice with MUC1 peptides coupled to anti-DNGR-1 inhibits the growth of MUC1-A2K/b-expressing tumours. Mice were injected with 2×10^5 MC38-MUC1-A2K/b tumours and 4 and 14 days later, the mice were given the ALG and LLLL peptides coupled to anti-DNGR-1 or to isotype control in the presence of anti-CD40 and poly-IC. (A) Experimental schedule. (B) Plots of tumour-free survival are shown for five DNGR-1 mice, five isotype control mice and five PBS mice; statistical analysis between the anti-DNGR-1 and isotype control groups was performed using the Mantel–Cox test. (C) Growth of tumours in individual mice is shown from a single experiment representative of two performed. (D) Size of tumours at day 14 combining the results of two independent experiments. *p* Values were determined using an unpaired *t*-test.

human DCs found in spleen, tonsil and blood, which is BDCA-3$^+$ and DNGR-1 positive, has now been identified [22], and these can further be defined as being CD11b$^-$ and dependent on the transcription factor BAFT3 [16, 22]. We isolated BDCA-3$^+$ cells from the peripheral blood of healthy volunteers and used an Ab to human DNGR-1 to target an MUC1 HLA-A2 peptide to the relevant DCs. The secretion of IFN-γ was observed in response to stimulation with the peptide, which was specific to the BDCA-3 cells originally pulsed with the anti-DNGR-1 Ab. Although the response was moderate, it was observed in four independent experiments using two donors, confirming the relevance of the murine results to the human situation. Importantly, we also showed that naïve T cells, stimulated with anti-DNGR-1 targeted peptide using the regime described, were able to recognised two different tumour cell lines expressing endogenous MUC1 (Fig. 4B and C). Although recent reports show that there are a number of human DC subsets capable of cross-presentation [35, 36] and direct comparison with the mouse system is difficult, the results illustrate the efficiency of DNGR-1 targeting as some previous studies using the same peptide and monocyte-derived DCs as stimulators failed to show recognition of tumour cell expressing endogenous MUC1 [26].

MUC1 is a membrane-bound mucin over-expressed in many carcinomas making a vaccine strategy targeting this glycoprotein relevant to a number of different cancers. It can be found expressed by over 90% of breast, ovarian and pancreatic carcinomas as well as many other adenocarcinomas. MUC1 has a number of characteristics that make it uniquely suited as a target for immunotherapy. Firstly, although expressed by normal epithelial cells it is dramatically up-regulated by 10- to 100-fold in the carcinomas that develop from them. Secondly, in normal epithelial cells, MUC1 is found on the apical surface and so extends into the ductal lumen where there is poor accessibility to tissue infiltrating T cells. However, in carcinomas when cell polarity is lost, MUC1 is expressed all over the cell. Thirdly, the O-linked glycosylation of MUC1 is drastically altered in cancer when truncation of the O-linked glycans is observed [37]. This may be relevant to the presentation of class I peptides as reduced glycosylation may make MUC1 more sensitive to protease digestion and therefore more efficient processing in the class I pathway. Moreover, there is increasing evidence that MUC1 is involved in tumour growth and antagonism of MUC1 signalling can inhibit this growth [38].

We have used a mouse model that allows the evaluation of the efficacy of targeting human MUC1 peptides presented by HLA-A2, the MHC class I type expressed by around 40% of the Caucasian population. Moreover, the mice are transgenic for human MUC1 and so the mice are tolerant to this mucin. In this model, MUC1 is expressed from its own promoter [28] and this results in the profile, levels of expression and glycosylation of MUC1 being very similar to that found in humans [28]. Thus, this model is a stringent clinically relevant model to evaluate this vaccine strategy. The results obtained suggest that targeting MUC1 peptides to DNGR-1 found on CD8α$^+$ DCs and their human equivalents is an effective strategy for inhibiting MUC1$^+$ tumour growth.

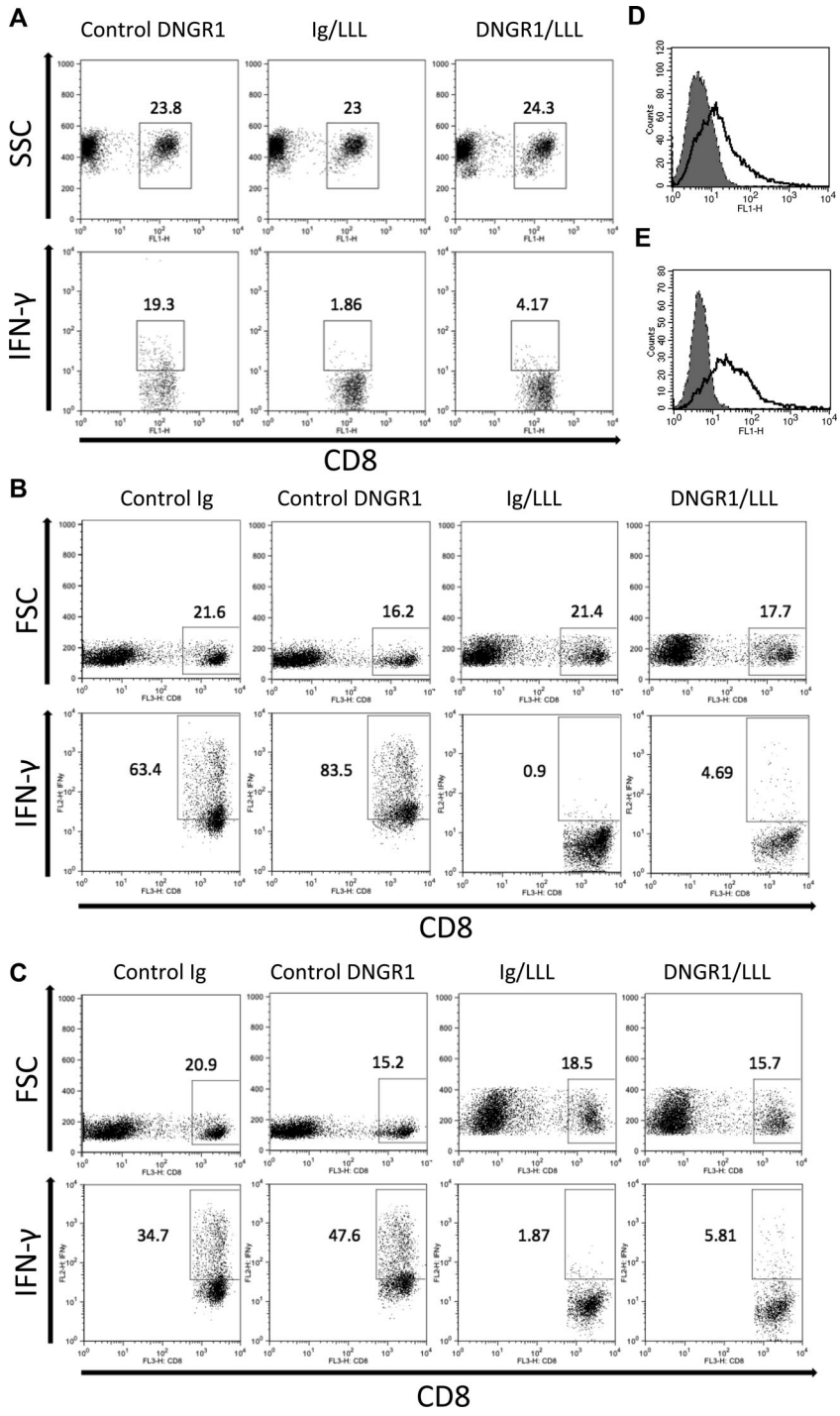

Figure 4. MUC1 peptides couple to anti-human DNGR-1 can induce $CD8^+$ T cells in vitro. BDCA-3^+ cells were isolated from PBMCs and incubated with the LLL peptide coupled to anti-human DNGR-1 or isotype control, in the presence of anti-CD40 and poly-IC, for 1 hour before incubating with autologous T cells for 7 days. The cells were then pulsed with peptide following a further 7 days incubation stimulated with autologous monocyte-derived DCs pulsed with peptide. (A) Seven days later, the cells were pulsed for 4 hour with LLL peptide and IFN-γ secretion by $CD8^+$ T cells measured by IFN-γ Secretion Assay. An aliquot of anti-DNGR-1-peptide stimulated cells was incubated for 4 hour with 5 μg/mL PHA and 1 μg/mL anti-human CD3 (Control DNGR-1). Data shown are representative of four independent experiments. (B, C) Cells were also pulsed with (B) MCF-7 cells and (C) Capan-1 cells and IFN-γ production was measured by intracellular cytokine staining. An aliquot of either isotype control (Control Ig) or anti-DNGR-1 (Control DNGR-1) stimulated cells was incubated for 4 hour with PBMC:Dynabeads® human T-Expander CD3/CD28. All the cells were analysed after gating on live cells. (D, E) Expression of endogenous MUC1 by (D) Capan-1 cells and (E) MCF-7 cells was determined by HMFG2 binding (black-line histogram) as assessed by flow cytometry. (Filled histogram, isotype control.)

Materials and methods

Cells

The MC38 cell line expressing human MUC1 was transfected with A2K/b in pSV [35] using Lipofectamine 2TX (Invitrogen). Transfected cells were sorted by flow cytometry for MUC1 and A2K/b expression using HMFG2 (MUC1) and BB7.2 (HLA-A2) Abs. The human breast cancer cell line MCF-7 and pancreatic cancer cell line Capan-1 were cultured in DMEM and 10% FCS.

Mice

MUC1 transgenic mice, where MUC1 expression is driven by its own promoter [28] on a pure C57BL/6 background, were crossed with A2K/b mice [29] also on a C57BL/6 background. For the

experiments described, F1 mice were used that are heterozygote for MUC1 and A2K/b. All animal experiments were conducted in line with Home Office guidance under Project licence PPL 70/6847.

Coupling of MUC1 peptides to anti-DNGR-1

HPLC-purified MUC1 peptides with a cysteine-HN$[CH_2]^2$NH-biotin at the C-terminus were obtained from ProImmune (Oxford, UK). Anti-mouse-DNGR-1 mAbs (Clone 7H11 [21]), anti-human-DNGR-1 mAbs (Clone clone 8F9 [21]) and their respective isotype controls (Rat IgG1 and mouse IgG2a from R&D Systems) in PBS were treated with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate for 30 min at room temperature to generate sulfo-reactive groups in tertiary amines. The activated Ab was purified by molecular size exclusion chromatography (Pierce Biotechnology), and modified MUC1 peptides were added (5:1 or 50:1 molar ratio), and the conjugation reaction was allowed to proceed for 1 hour at 37°C. Conjugated Abs were purified using a GammaBind Plus Sepharose (GE Healthcare) column to remove free peptide. The extent of biotinylation of the mAb was assessed to quantify the amount of peptides bound per molecule of Ab, using the FluoReporter kit (Invitrogen) as per manufacturer's instructions.

Vaccination of mice to evaluate immune response

MUC1xA2Kb transgenic mice were injected subcutaneously into both flanks (50 μL per flank) with MUC1 peptides coupled to anti-mouse DNGR-1 mAb or coupled to IgG1 isotype control, together with endotoxin-free anti-CD40 (12.5 μg per 100 μL, BD Pharmingen) and poly-IC (5 μg per 100 μL, InvivoGen). The spleens were removed after 7 days following the first injection or 7 days following a further two injections given 2 weeks apart. Ficoll-purified splenocytes, 1×10^6 cells in 100 μL RPMI 5% FCS, were incubated with 1 μg of peptides used as the immunogen, or control (non-specific peptide) for 48–60 hours at 37°C. The supernatants were assayed for IFN-γ secretion by ELISA according to the manufacturer's instructions (R&D Systems). All stimulations were performed in triplicate.

Inhibition of tumour growth

In the prophylactic setting, mice were vaccinated with three subcutaneous injections of MUC1 peptides coupled to anti-mouse DNGR-1 or isotype control in the presence of 12.5 μg per 100 μL anti-CD40 and 5 μg per 100 μL poly-IC. The vaccination was repeated twice, with 2 weeks between each injection. Seven days following the last injection, 5×10^5 MC38 tumour cells expressing MUC1 and A2K/b (MC38-MUC1-A2K/b) were injected subcutaneously into the left flank.

In the therapeutic setting, 5×10^5 MC38-MUC1-A2K/b tumour cells were injected subcutaneously, and 4 and 14 days later, the mice were injected with MUC1 peptides coupled to anti-DNGR-1 or isotype control Abs in the presence of anti-CD40 and poly-IC. Tumour growth was assessed by external caliper measurements and volumes calculated as $(a \times b^2)/2$, where a is the largest diameter and b is the smallest.

Stimulation of human T cells in vitro

Total PBMCs were isolated from 100 mL of blood of healthy volunteers with Ficoll-paque PLUS (GE Healthcare) as per manufacturer's instructions. All blood was taken with permission of the South East London REC1 Committee, ethics approval number 09/H0804/92. Myeloid DCs expressing BDCA-3 were separated from total PBMC using CD141 (BDCA-3) microbeads kit (Miltenyi Biotech) as per manufacturer's instructions. The BDCA-3 positive population was stimulated with either anti-DNGR-1 mAbs or IgG2a control coupled to LLLTVLTVV-cysteine-HN$[CH_2]^2$NH-biotin in 1 mL PBS containing 0.5% BSA, 10 μg anti-CD40 and 25 μg poly-IC, for 1 hour at 37°C. Stimulated BDCA-3 positive cells were returned to autologous BDCA-3 negative selected PBMCs at a ratio between 1:80 and 1:100, and cultured in serum-free media IMV-V (Life Technologies) containing 20 ng/mL human IL-7 (R&D Systems) and 50 μM β-mercaptoethanol (Sigma). Seven days later, cells were stimulated for 4 hour at 37°C with the peptide LLLTVLTVV, and after 14 days at a ratio of 1:10 with peptide-pulsed autologous monocyte-derived DCs. Autologous monocytes were isolated using CD14 microbeads (Miltenyi Biotech) and cultured for 7 days with 1500 U/mL human IL-4 and 400 U/mL human GM-CSF (both from R&D Systems) in AIM-V media (Life Technologies).

On day 21, the cells were given a final stimulation with peptide and 4 hour later secretion of IFN-γ measured using the IFN-γ Secretion Assay (Miltenyi Biotech) as per manufacturer's instructions. As a positive control for the assay, aliquots of T cells were incubated with a mixture of 5 μg/mL PHA (Sigma) and 1 μg/mL anti-human CD3 (clone UCHT1; Millipore).

PBMC from different donors were stimulated in vitro as described and on day 21 the induced T cells were given a final stimulation with MCF-7 or Capan-1 MUC1-expressing cancer cell lines (1:1 cancer cell lines:T cells) that had been treated for 3 days with 300 U/mL of IFN-γ. After a 4-hour incubation, the production of IFN-γ was measured by intracellular cytokine staining as previously described (Correa et al. [26]). As a positive control for the assay, an aliquot of T cells was incubated with PBMC:Dynabeads® human T-Expander CD3/CD28 (Life Technologies) at a 1:1 ratio (cell:bead) instead of cancer cells.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviation: **CTL**: cytotoxic T lymphocyte

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