Citation: Molecular Therapy — Oncolytics (2016) **3,** 15023; doi:10.1038/mto.2015.23
Official journal of the American Society of Gene & Cell Therapy

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ARTICLE

Design and characterization of the tumor vaccine MGN1601, allogeneic fourfold gene-modified vaccine cells combined with a TLR-9 agonist

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The tumor vaccine MGN1601 was designed and developed for treatment of metastatic renal cell carcinoma (mRCC). MGN1601 consists of a combination of fourfold gene-modified cells with the toll-like receptor 9 agonist dSLIM, a powerful connector of innate and adaptive immunity. Vaccine cells originate from a renal cell carcinoma cell line (grown from renal cell carcinoma tissue), express a variety of known tumor-associated antigens (TAA), and are gene modified to transiently express two co-stimulatory molecules, CD80 and CD154, and two cytokines, GM-CSF and IL-7, aimed to support immune response. Proof of concept of the designed vaccine was shown in mice: The murine homologue of the vaccine efficiently (100%) prevented tumor growth when used as prophylactic vaccine in a syngeneic setting. Use of the vaccine in a therapeutic setting showed complete response in 92% of mice as well as synergistic action and necessity of the components. In addition, specific cellular and humoral immune responses in mice were found when used in an allogeneic setting. Immune response to the vaccine was also shown in mRCC patients treated with MGN1601: Peptide array analysis revealed humoral CD4-based immune response to TAA expressed on vaccine cells, including survivin, cyclin D1, and stromelysin.

Molecular Therapy — Oncolytics (2016) **3**, 15023; doi:10.1038/mto.2015.23; published online 10 February 2016

INTRODUCTION

Tumor vaccines restore the immune system's intrinsic ability to recognize tumor cells. Cell-based tumor vaccines are classified as dendritic cell (DC)-, T-cell- or tumor cell-based vaccines. For the latter one, tumor-specific effects were shown including activation of T cells and strengthening of $CD8^+$T-cell responses by direct antigen activation and cross-priming, development of memory cells, and increase of antibody-based response.[1,2] Cell-based tumor vaccines own a huge reservoir of tumor-related antigens and are, therefore, able to address a broad repertoire of T cells. Plurality of antigens on cell-based vaccines hampers immune escape of the tumor cells by selective antigen loss. However, individual antigens of the cell-based vaccines evoke a rather weak immune response underlining the necessity to strengthen their immunogenicity by multiple gene modifications.

Preparation of multiple gene-modified cells requires high-efficient expression vectors. Recently, MIDGE (minimalistic immunogenically defined gene expression) DNA vectors were developed for clinical use[3–5] allowing the generation of multiple gene-modified cells with only minimal amount of foreign DNA. MIDGE vectors display a linear covalently closed topology with single-stranded loops and are biotechnologically manufactured from plasmids. Their small size of about 1,200 bp plus coding sequence is based on their exclusive content of the expression cassette consisting of the CMV promoter, the selected coding sequence, and a poly(A) signal. The prevention of genes for resistance to antibiotics, of replication origins, and other functional elements improves their overall safety profile and ensures that application of MIDGE vectors does not add conflicting potential to public health issues.

Transient gene modification of cells helps to maintain their natural expression profiles by minimizing adaptation processes following otherwise stable transfection. Additionally, waiving clonal selection preserves the heterogeneity of cells representing all subtypes outgrown from original tumor tissue. Preservation of the antigen repertoire of the source cell line during the manufacturing process to vaccine cells is important because this repertoire is a main criterion for selection of source cells.

Besides the selection of cell line, vector, and gene modification, the identification of an additional immunomodulator as multiplier of the tumor-specific immune response is crucial. Toll-like receptor 9 (TLR-9) agonists are powerful connectors of innate and adaptive immunity and therefore supposed to be ideally suited to strengthen tumor vaccines.[6] dSLIM (**d**ouble **s**tem **l**oop **i**mmuno**m**odulator) is a noncoding dumbbell-shaped and covalently closed DNA molecule with nonmethylated CG motifs acting via TLR-9 (refs. 7–9). Currently, dSLIM is evaluated in clinical trials for the treatment of solid tumors.[10,11]

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Received 14 August 2015; accepted 1 December 2015

Renal cell carcinoma (RCC) is well known for its response to immune therapies.[12,13] Launch of targeted therapies has improved treatment of patients with metastatic RCC. However, RCC, especially at metastatic stage, remains a life-threatening condition with high medical need for effective treatment.

The aim of the current work was to develop a tumor vaccine for an improved treatment of metastatic renal cell carcinoma (mRCC). During our previous development of tumor vaccines, cells derived from autologous tumor tissue were gene modified to discretely express IL-2, IL-7, IL-12, or granulocyte-macrophage colony-stimulating factor (GM-CSF).[14–16] The use of twofold gene-modified vaccine cells secreting IL-7 and GM-CSF presented a new milestone in autologous vaccine development.[17] In this study, 50% of treated patients showed clinical response to treatment with at least disease stabilization. Encouraged by these results and driven by the increased knowledge and immunological understanding of tumor vaccines,[18] additional gene modification of vaccine cells (besides cytokines) with co-stimulatory molecules CD80 (ref. 19) and CD154 (refs. 20,21) was chosen. These modifications were supposed to further improve immune response to the vaccine antigens by mimicking features of antigen presenting cells (APC) and T cells and result in fourfold gene-modified cells.

The concept of fourfold gene-modified cells was evaluated in mice, using a murine vaccine consisting of murine tumor cells and expressing murine cytokines and co-stimulatory molecules in combination with the TLR-9 agonist dSLIM. Proof of concept of the antitumor efficacy was shown as well as cellular and humoral immune responses. Multiple toxicological studies did not reveal any safety concerns. The next developmental step was the identification a human cell line for generation of a human vaccine. B25MOL cells were selected as source material for the vaccine due to their success in an autologous treatment schedule of an mRCC patient (complete response for 7 years, survival for 13 years) and their broad expression of tumor-associated antigens (TAA). Fourfold (CD80, CD154, GM-CSF, and IL-7) gene-modified vaccine cells were combined with the TLR-9 agonist dSLIM—resulting in MGN1601. Expression characteristics of vaccine cells were analyzed. The immune responses shown in murine studies were confirmed in translational analyses with blood samples from patients treated within the ASET (**A**ssess **S**afety and **E**fficacy of the **T**umor Vaccine MGN1601) study with MGN1601.

RESULTS

Efficacy of the designed vaccine in murine tumor models (syngeneic setting)

In order to evaluate the efficacy of the designed vaccine for treatment of RCC, a murine homologue of the vaccine was manufactured. This vaccine consisted of fourfold gene-modified murine RCC cells (Renca), combined with dSLIM.

Use as prophylactic vaccine. First, the immunotherapeutic concept was assessed by prophylactic vaccination in a syngeneic mouse model. Balb/c mice were treated four times with the murine homologue before tumor growth was initiated by inoculation of Renca cells (Figure 1a,b). All mice of the vaccinated group survived, and none of them developed a tumor at any time of the experiment (Figure 1a). In contrast, 70% of mice treated with phosphate-buffered saline (PBS) developed a tumor, and none of the tumor-bearing mice survived the experimental period due to tumor growth. In vaccinated mice, tumor-free survival as well as total survival were significantly increased ($P < 0.001$; Figure 1b). These data unequivocally proved that prophylactic vaccination with the newly designed vaccine protects mice from tumor development after inoculation of Renca cells.

Use as therapeutic vaccine. The newly designed concept was further evaluated by therapeutic vaccination using different variants of the vaccine (murine homologue). Balb/c mice first received Renca cells for tumor inoculation. One week later, treatment was started (Figure 1c,d and Supplementary Table S1). Treatment was repeated three times ($n = 12$ mice/group). One important goal of the study was to evaluate the efficacy of experimental variants of the vaccine to inhibit tumor growth and thus the necessity of the components within the therapeutic concept. Complete vaccine ("complete") was compared to the efficacy of vaccine cells without the immunomodulator dSLIM ("tf Renca" ≙ fourfold gene-modified Renca cells), to irradiated Renca cells ("Renca" ≙ without gene modification, without dSLIM), and to dSLIM as single component ("dSLIM").

Mean tumor volume at the end of the study or at the date of death of a mouse was 1,370 mm^3 for mice receiving PBS (Figure 1c), whereas mice treated with the murine homologue showed an average tumor volume of 69 mm^3 (11 mice without tumor and 1 mouse with 832 mm^3 tumor volume). Experimental variants of the vaccine used resulted in mean tumor volumes between those of PBS- and vaccine-treated mice. Tumor volumes of mice treated with "complete vaccine" or with "tf Renca" were statistically significant lower ($P < 0.001$) compared to those of PBS-treated mice. Furthermore, tumor volumes of mice treated with "dSLIM" as monotherapy were significant lower ($P < 0.05$), whereas tumor volumes of mice treated with "Renca" showed no significant reduction. These data are summarized in Supplementary Table S1.

Tumor growth in tumor-bearing mice of all five treatment groups was compared over the time course of the study (Figure 1d and Supplementary Figure S1). All vaccinated animals showed reduced tumor growth compared to PBS-treated mice. However, the complete vaccine achieved the most prominent effects. Furthermore, the survival rate of tumor-bearing mice was highest (92%) for mice treated with the complete vaccine, whereas all tumor-bearing mice treated with PBS died during the study due to tumor-related reasons. Survival data of mice in the other three treatment groups ranked between those of PBS and complete vaccine groups: "tf Renca": 75%, "Renca": 50%, and "dSLIM": 17%.

Mice that had been classified as tumor bearers during the study but finished the study without measurable tumor size were classified as "complete remission." Eleven out of 12 tumor-bearing mice of the complete vaccine group were free of tumor at the end of the study, resulting in 92% complete remission rate. In contrast, none of the PBS-treated mice showed any reduction of tumor volume at any time point. The variants of the vaccine showed intermediate complete remission rates: "tf Renca": 66%, "Renca": 50%, and "dSLIM": 17%.

In summary, the newly designed therapeutic concept was identified as superior over other tested experimental variants underlining the relevance and potential synergistic effects of the vaccine cells, their gene modification, and the TLR-9 agonist dSLIM.

Immunomodulating effects of the vaccine in mice (allogeneic setting)

Next, the immunomodulatory effects of the vaccine were addressed. Again, the murine homologue of the vaccine was repeatedly applied (8 to 13 times) once weekly to NMRI mice (Naval Medical Research Institute mouse strain, allogeneic to Renca cells).

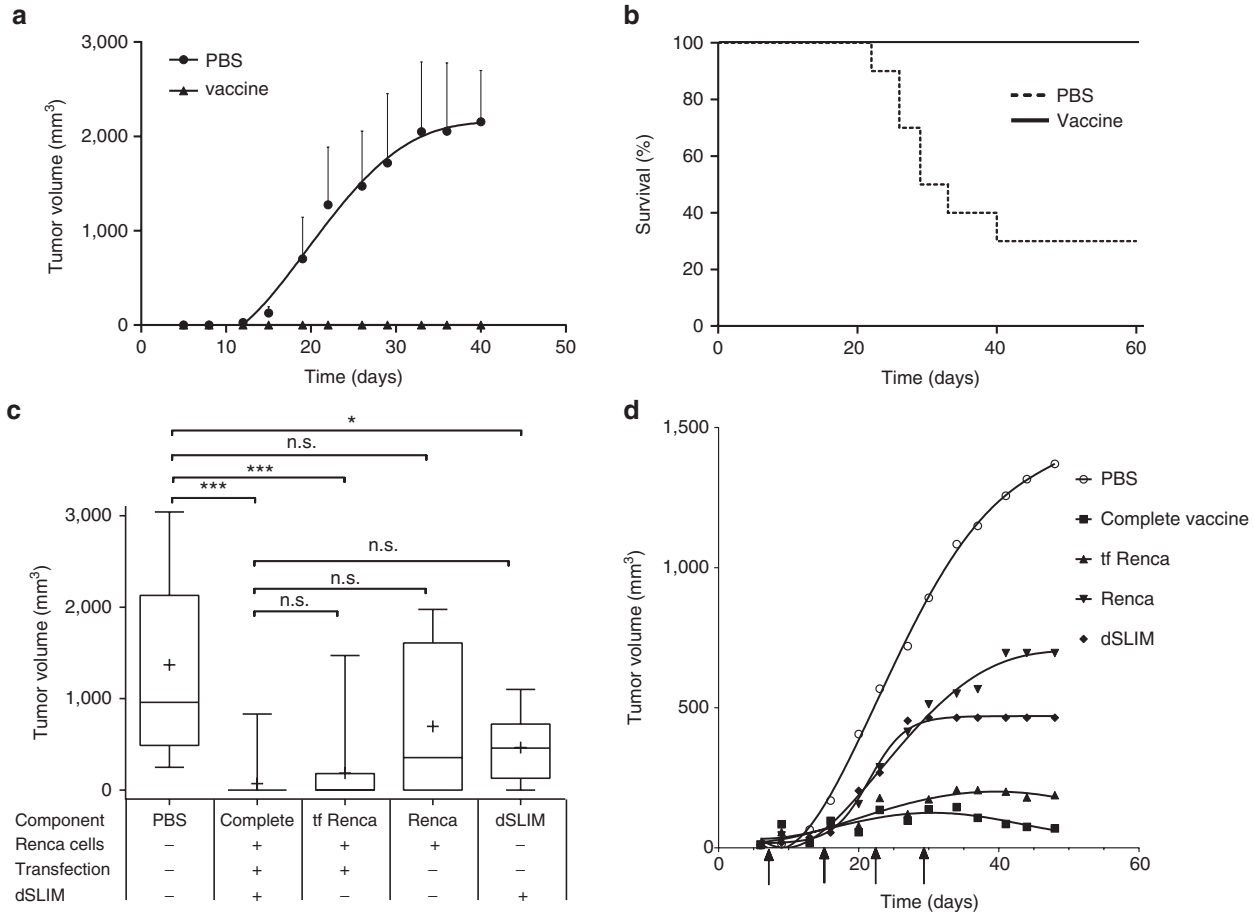

Figure 1 Vaccination studies using the murine vaccine in Balb/c mice (syngeneic setting). (**a** and **b**) Prophylactic vaccination study. Mice received four weekly injections of the murine homologue of the vaccine ($n = 10$) or PBS ($n = 10$). Thereafter, tumor inoculation with Renca cells was performed. (**a**) Tumor volume of tumor-bearing mice, given as mean + SD and (**b**) survival of all mice was assessed for 60 days after tumor inoculation. A representative study is shown. (**c** and **d**) Therapeutic vaccination study using a range of experimental variants of the vaccine. Mice received tumor inoculation with Renca cells and, subsequently, 4 weekly injections with vaccine variants ($n = 12$ mice/group). (**c**) Mean (+) and median tumor volume, 5–95% confidence intervals, + SD for each treatment group are shown. For calculation of mean tumor size, the tumor volume of surviving mice at day 60 as well as the tumor size of nonsurvivors measured before death was used. Statistical analysis was done by one-way analysis of variance followed by Tukey's test ***$P < 0.001$, *$P < 0.05$, n.s., not significant; $P > 0.05$ (versus PBS-group). (**d**) Tumor growth. Average tumor size per time was calculated from all tumor-bearing mice (6–12 mice) of the respective treatment group. Arrows indicate vaccinations.

Attraction of APC and T cells to the application site. Skin sections of the injection sites of the vaccine were analyzed for the presence of T cells ($CD4^+$ or $CD8^+$ cells) and APC ($CD86^+$ cells) in NMRI mice. A bright staining intensity for $CD4^+$ and $CD8^+$ T cells, as well as for APC ($CD86^+$ cells) was seen in mice treated repeatedly (eight times) with the vaccine, while skin sections of application sites of PBS-treated mice did not stain for any of the analyzed cell populations (Figure 2a and Supplementary Figure S2). These findings indicate the recruitment of T cells and APC by the vaccine to the injection sites.

Induction of vaccine specific antibodies. Sera of vaccinated mice were analyzed for Renca-specific antibodies after eight applications of the vaccine (Figure 2b). Sera of all vaccinated mice clearly recognized Renca cell lysates (titer 11,000 to 35,000), while sera of PBS-treated mice did not react at all, indicating high $CD4^+$-dependent B-cell responses to the vaccine.

Induction of vaccine-specific cytotoxicity. Mice were vaccinated repeatedly (13 times) with either low dose or high dose of the vaccine (murine homologue) in an allogeneic setting. Thereafter, spleen cells were isolated, re-stimulated with Renca cells *ex vivo*, and analyzed for specific cytotoxicity against Renca cells as target (Figure 2c). Highest Renca-specific cytotoxicity of spleen cells was found in the high-dose group, although all examined groups showed pronounced lytic activities for Renca cells if compared to spleen cells from PBS-treated mice. Cytotoxicity of spleen cells was maintained even 4 weeks after the last high-dose application (recovery group), indicating a sustained $CD8^+$-based immune response against the vaccine cells.

Evaluation of safety

To assess the safety of the tumor vaccine, single- and repeated-dose toxicological studies were performed using either MGN1601 in rats (heterologous model) or the murine homologue in mice (homologous model, allogeneic setting) according to regulatory requirements. In the heterologous setting, the single administration of up to a 500-fold excess regarding the human dose (per kg body weight) as well as the repeated application (five times) of up to a 60-fold excess did not result in any relevant toxicological findings. In the homologous model, the murine vaccine was applied to NMRI mice. Repeated application (13 times) of up to a 3,000-fold excess regarding the human dose resulted in only minor observations

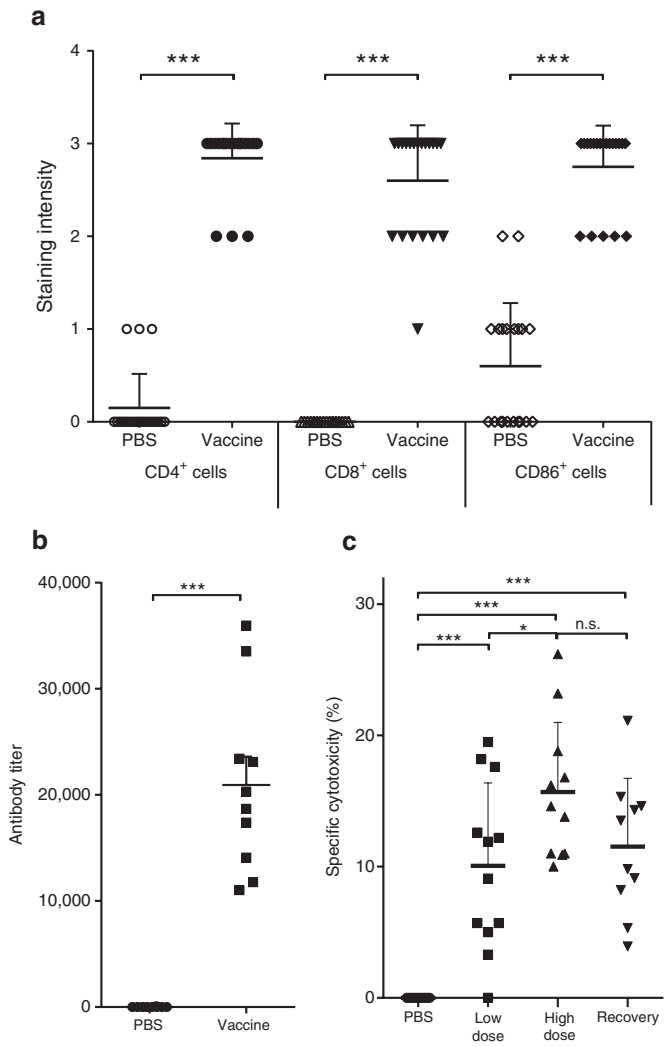

Figure 2 Immune response to treatment of NMRI mice with the murine vaccine (allogeneic setting). (**a**) Staining intensity of skin sections at the application site of the murine vaccine after 8 weekly injections. Skin sections of the application sites were analyzed by immunohistochemistry for the presence of $CD4^+$-, $CD8^+$-, and $CD86^+$- cells. Staining intensity was recorded as 0 for "no staining," as 1 for "slight staining," as 2 for "moderate staining," and as 3 for "pronounced staining." Two injection sites per animal were analyzed, resulting in 20 analyses per parameter. Analytical results and mean + SD are shown. Statistical analysis was done by Student's *t*-test; ***$P < 0.001$. (**b**) Titer of antibodies reactive to Renca cells after 8 weekly vaccinations. One week thereafter, serum was gained and analyzed by enzyme-linked immunosorbent assay for antibodies reactive to Renca lysate. Dilution of serum is given as titer. Analytical results as well as mean + SD are shown. Statistical analysis was done by Student's *t*-test; ***$P < 0.001$. (**c**) Cytotoxicity of spleen cells of mice repeatedly vaccinated with low dose (12 mice) or high dose of the murine vaccine (22 mice) or PBS (12 mice). One week (recovery group: 5 weeks) after the 13th application, spleen cells were isolated and analyzed for cytotoxicity toward Renca cells. The respective value of PBS-treated mice was subtracted. Statistical analysis was done by one-way analysis of variance followed by Tukey's test. *$P < 0.05$ (versus high dose of the vaccine); ***$P < 0.0001$ (versus PBS). Given are the analytical results plus mean + SD.

representing most probably immune activation due to the anticipated pharmacodynamic effects of the vaccine. Observations included a reversible increase of 35% (statistically significant with $P < 0.01$, only for female mice) in absolute and relative spleen weight (Supplementary Figure S5) as well as lymphoid hyperplasia of the lymph nodes. Additionally, histopathology of the injection sites revealed infiltration by mononuclear cells and granulomatous inflammation. No signs for systemic toxicity of MGN1601 or its murine homologue were observed.

Selection and characterization of B25MOL cells as basis for MGN1601

After proof of concept of the designed vaccine was shown in mice, the next aim was the transition of the murine to a human vaccine for mRCC patients. This translational effort required the identification of a suitable cell line. In order to assure a comprehensive presentation of TAA by the vaccine cells, the cell line used as source for vaccine preparation was carefully selected. Selection was done on the basis of 17 cell lines each established from tumor material of patients with RCC obtained during nephrectomy. These cell lines had been used for the preparation of autologous vaccines. Based on propagation characteristic and clinical response, cell line B25MOL was chosen for further development of an allogeneic tumor vaccine. B25MOL cells originate from tumor material of a female patient with clear cell mRCC (stage IV with multiple metastases (pT3a pTx G3)). Tumor tissue showed outgrowth of cells after 6 weeks of cultivation, and cells exhibited stable propagation. No clonal selection was performed. After four autologous vaccinations with gene-modified B25MOL cells expressing IL-7 and GM-CSF over a course of 6 weeks, the patient showed complete clinical response (lasting for 7 years and resulting in 13 years of survival) with disappearance of all metastases indicating immunologically favored characteristics of these cells. A master cell bank of B25MOL cells was manufactured, stored, and certified for use in humans. Stability of transcriptome was analyzed by comparison of RNA of B25MOL cells after initial and late round of seeding of a master cell bank aliquot (Figure 3a). From 32,000 genes analyzed, only 5 changed expression more than threefold between passage 20 and passage 40. Three of these genes were not annotated at the time of analysis and two coded for small nucleolar RNA (SNORA36C and SNORA36A) involved in the modification of 18S rRNA. mRNA data of the cells used as source for the vaccine showed expression of TAA (see Supplementary Table S2). Congruency of mRNA data and protein expression data was evaluated for 242 surface proteins, easily accessible for fluorescence-activated cell sorting. Besides a few exceptions, correlation was roughly given. Out of the analyzed antigens, 102 were clearly expressed (geometric mean value higher than threefold of respective isotype control). From those, about a dozen are described as immunogenic or are even in use as components of tumor vaccines, including CD40, CD44, CD54 (ICAM), CD73, CD95 (FAS), CD105 (TGF-β receptor complex, endoglin), CD146 (MCAM), CD151, CD227 (MUC1), CD326 (EpCAM), CD340 (Her-2/neu), EGFR, and Met (Figure 3b).

Gene modification of B25MOL cells

Four different MIDGE vectors each coding for the expression of CD80, CD154, GM-CSF, or IL-7 were used for transient gene modification of B25MOL cells. Thereafter, gene-modified B25MOL cells were gamma-irradiated to prevent further propagation of cells.

Typically, about 50% of vaccine cells expressed CD80 as well as CD154 (Figure 4a) on their cell surface. Concomitantly, vaccine cells secreted the cytokines GM-CSF and IL-7. Both accumulated in the culture supernatants and were quantified to 38,035 ± 9,266 (GM-CSF) and 3,815 ± 703 (IL-7) pg/1 × 10^6 cells/4 hours. Secretion data of cytokines are given as mean (± SD) of eight batches ($N = 8$), representing >2 × 10^{10} vaccine cells. Concentration of GM-CSF was

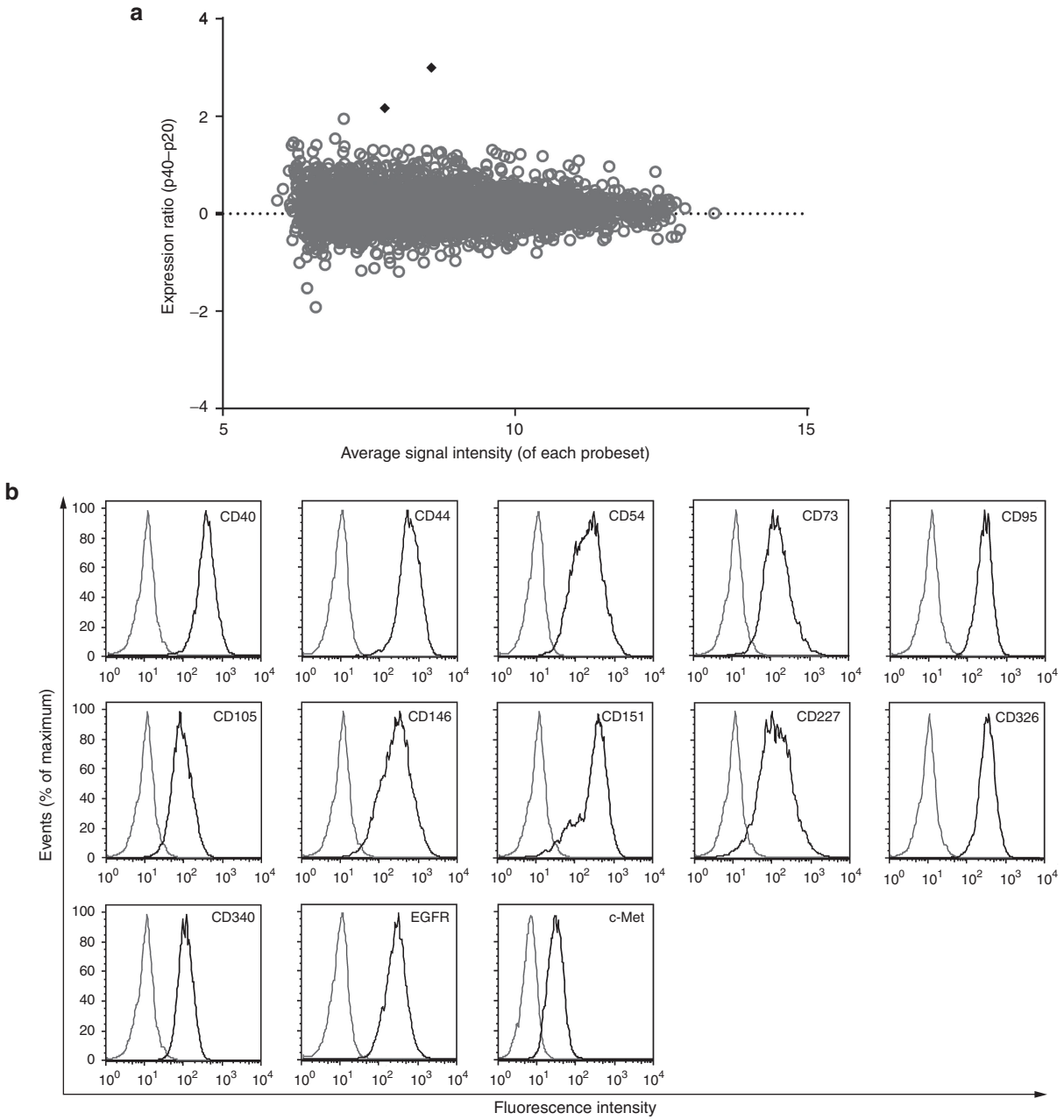

Figure 3 mRNA and protein expression data of B25MOL cells. (**a**) Stability of mRNA expression during cultivation of B25MOL cells via microarray. Passage 20 and 40: two biological replicates per analyzed passage, 10 microarrays in total are compared. The expression ratio (log base 2) of each spot is plotted against its average expression intensity of the probe sets. Dark diamonds identify the two differentially expressed annotated genes (SNORA36C and SNORA36A). Analysis of mRNA data was performed by Robust Multiarray Average preprocessing and Linear Model for Microarray data. Differential expressed genes are defined to show at least threefold changes of expression and a *P* value of moderate *t*-statistic of 0.05 at maximum. See also Supplementary Table S1. (**b**) Expression of selected cell surface antigens on B25MOL cells via flow cytometry. Histograms of antibody stained B25MOL cells (bold black lines) and corresponding isotype controls (gray lines) are shown. A representative histogram for each antigen is shown. At least 5 (up to 13) independent experiments were performed.

about 10 times higher than that of IL-7, indicating different secretory potentials for the two cytokines by the vaccine cells.

A 6-day culture of vaccine cells confirmed the higher secretory capacity of GM-CSF (Figure 4b). The concentration of IL-7 in the supernatant increased for about 1 day and was stable thereafter, while the concentration of GM-CSF increased pronounced within the first 24 hours and moderate thereafter. Furthermore, the expression of CD80 on the surface of living cells remained almost constant over several days. Due to the irradiation of gene-modified B25MOL cells, the percentage of living cells constantly dropped after a lag phase of about 10 hours until day 14 when no viable cell was detected.

Expression of cytokines was additionally analyzed *in vivo*. For this, a murine homologue of the vaccine (murine Renca cells, gene-modified with murine forms of CD80, CD154, GM-CSF, and IL-7, in combination with dSLIM) was manufactured. *In vitro* expression data were comparable to MGN1601 (Figure 4c, data of cytokine secretion: 25,295 pg/4 hours × 1×10^6 cells (± 10,852) for GM-CSF

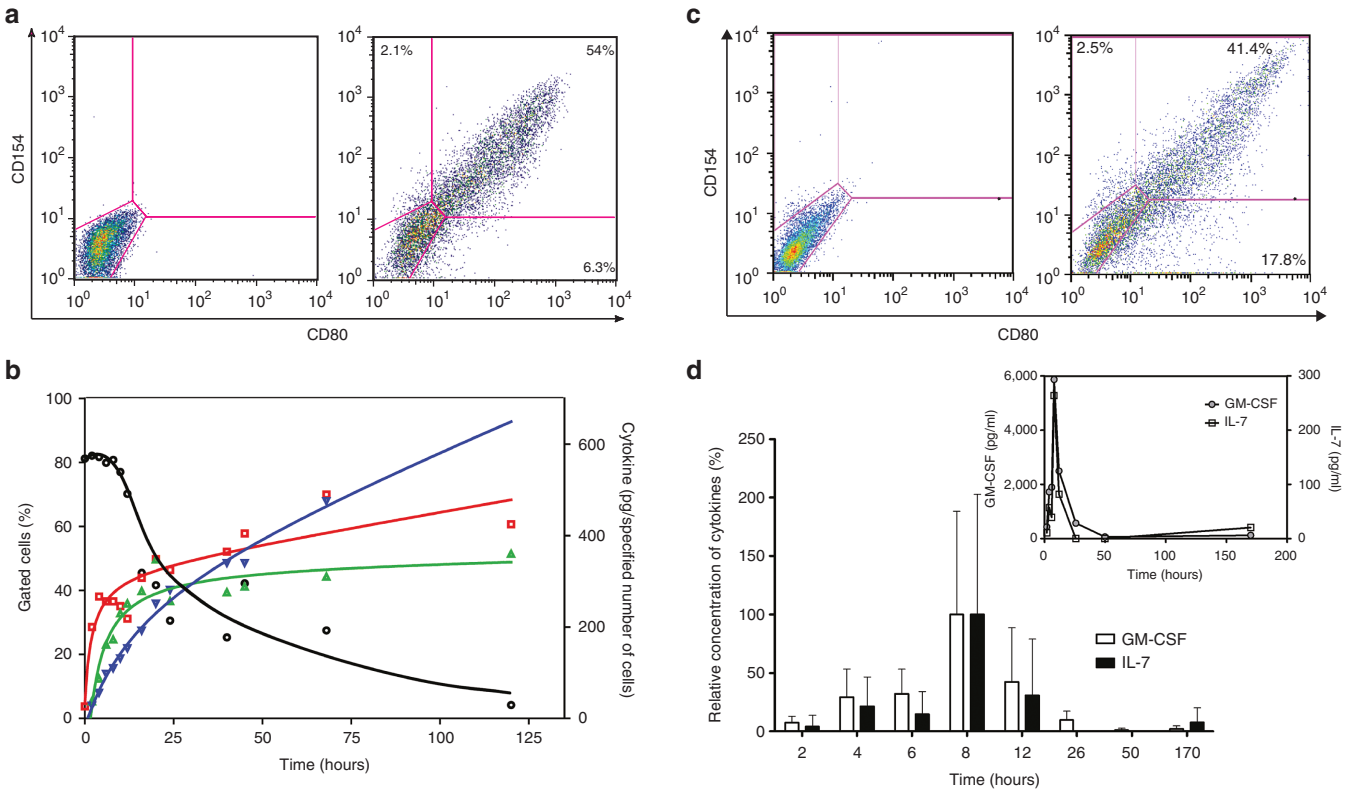

Figure 4 Expression analysis of vaccine cells. (**a**) Expression of CD80 and CD154 in vaccine cells of MGN1601: vaccine cells were cultivated for 4 hours, thereafter stained with CD80-FITC and CD154-APC (right) or the respective isotype controls (left) and analyzed by flow cytometry. Co-expression of CD80 and CD154 is shown in a representative dot plot of vital cells. The percentage of positive and vital cells within all cells (vital plus nonvital) is depicted within the respective gates. (**b**) Kinetic of expression *in vitro*: vaccine cells were cultivated for the indicated periods, up to 125 hours. Accumulation of cytokines (GM-CSF in blue, IL-7 in green) in the culture supernatant was analyzed by enzyme-linked immunosorbent assay. Concentration of cytokines IL-7 (pg/100,000 cells) and GM-CSF (pg/5,000 cells) is depicted on the right y-axis. Expression of CD80 (percentage within living cells, red) and the ratio of living cells (black) was analyzed by flow cytometry and is indicated on the left y-axis: gated cells (%). Due to irradiation of cells the percentage of viable cells decreases within days. (**c**) Expression of CD80 and CD154 in vaccine cells of the murine homologue of MGN1601: vaccine cells were cultivated for 4 hours, thereafter stained with CD80-FITC and CD154-PE (right) or the respective isotype controls (left) and analyzed by flow cytometry. Co-expression of CD80 and CD154 is shown in a representative dot plot of vital cells. The percentage of positive and vital cells within all cells (vital plus nonvital) is depicted within the respective gates. (**d**) Kinetic of expression of cytokines *in vivo*. Murine homologue of MGN1601 was applied to mice and the presence of GM-CSF and IL-7 in biopsy-homogenates of the injection sites was analyzed by enzyme-linked immunosorbent assay. Relative concentration is displayed as mean + SD of six biopsies (three animals per point in time, each left and right side). 100% was assigned to the mean value at 8 hours after application. The inset shows the absolute concentration of GM-CSF (left y-axis) and Il-7 (right y-axis). For the preparation of homogenates, the tissue samples were diluted for about factor 5 with PBS. Therefore, the concentration of the cytokines *in vivo* is about 5 times higher than that measured in the homogenates.

and 660 pg/4 hours × 1 × 10^6 cells (± 143) for IL-7 ($N = 7$), representing about 5 × 10^9 vaccine cells). The murine homologue was injected s.c. to mice in an allogeneic setting. The concentration of the murine cytokines GM-CSF and IL-7 at the injection sites was analyzed in biopsy homogenates at various time points after application of the vaccine (Figure 4d). Cytokine concentration increased after application for up to 8 hours and decreased thereafter. Fluctuation in individual cytokine concentration is most probably due to the fact that biopsies do not perfectly co-localize with the site of highest cytokine concentration. The concentration of GM-CSF *in vivo* was clearly higher (10–20 times) than that of IL-7, confirming the *in vitro* secretion characteristics.

Comparison of expression characteristic of B25MOL cells and vaccine cells

B25MOL cells own a unique repertoire of potentially immunogenic TAA. Maintenance of this profile during their manufacturing process to vaccine cells is an important goal. Comparison of expression of cell surface proteins from B25MOL cells and engineered vaccine cells is shown in Figure 5. Geometric mean values of antigens on B25MOL cells and on vaccine cells are mainly similar, indicating constant expression of the analyzed surface proteins. Especially highly expressed proteins (geometric mean values > 100) show only marginal changes in their surface expression. Besides the expected increase in expression of CD80 and CD154 two ABC transporters show higher expression (7.4-fold for CD338 and 2.7-fold for CD243) as well as CD15, SSEA-1, and disialoganglioside. Reduced expression was only noted for CD201 and MUC1 (CD227). Both molecules got lost from the cell surface during manufacturing of B25MOL cells to vaccine cells, probably due to either a shedding process because their soluble forms are described[22,23] or an increase in intracellular compartments. Comparison of expression of selected surface-TAA in B25MOL cells and vaccine cells is shown in Figure 5b. Expression of TAA is highly preserved on vaccine cells indicating the maintenance of the characteristics of B25MOL cells in engineered vaccine cells. Furthermore, expression data of mRNA analyzed by microarrays remain mainly on similar levels in the vaccine cells compared to B25MOL cells (K. Heinrich, personal communication).

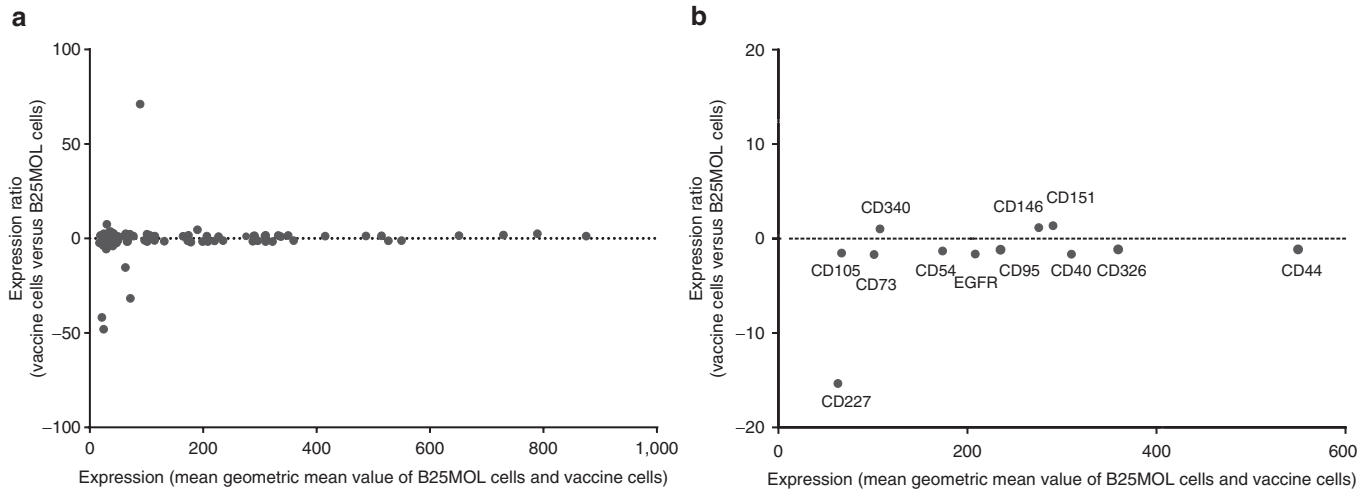

Figure 5 Comparison of surface antigen expression of B25MOL cells and vaccine cells. (**a**) Expression ratio of 242 human cell surface markers was compared by BD Lyoplate. Antigens with expression level exceeding three times the respective isotype control were identified and compared for both kinds of cells (120 antigens). (**b**) Expression ratio of 13 selected TAA was compared by flow cytometry. For individual antigens, this analysis was independently repeated three to six times. Quotient of geometric mean values (vaccine cells/B25MOL cells) is displayed on the y-axis, whereas on the x-axis, the mean geometric mean value of both kinds of cells for each antigen (after correction for the respective isotype controls) is shown.

Induction of TAA-specific antibodies by MGN1601 in patients

After assuring that the human vaccine expresses the analyzed TAA, co-stimulatory molecules, and cytokines, the translational course was continued, and a phase 1/2 clinical trial (ASET) was performed to assess safety, efficacy, and immunological effects of MGN1601 in mRCC cancer patients[24] (V. Grünwald, S. Weikert, I.G.H. Schmidt-Wolf, S. Hauser, A. Magheli, K. Kapp *et al*., personal communication). Here, the translational analyses regarding CD4-based humoral immune responses are in the focus in order to strengthen the developmental process from mice to men.

In short, 19 heavily pretreated patients with mRCC were included to receive eight doses of MGN1601 within 12 weeks (intended-to-treat)—with 10 of them completing the treatment per protocol (PP). Application of MGN1601 to patients was safe and well tolerated. Notably, the PP-treated patients showed significantly increased overall survival ($P < 0.05$, median overall survival at final study analysis of 115.3 weeks) over the intended-to-treat population.[24] Here, the humoral immune response of MGN1601-treated patients against TAA expressed on vaccine cells was evaluated. For this purpose, sera of the PP-treated patients from the ASET study were analyzed for their binding properties to 17 known TAA—each represented on an array by overlapping peptides (Table 1). The serum of each patient showed binding to peptides either not recognized by their respective pre-immune serum or with a clearly increased intensity after MGN1601 treatment (shown for a single patient in Supplementary Figure S3). In average, serum of each patient recognized 37 out of 4,399 peptides (±29, $N = 10$).

Each analyzed antigen was recognized by sera of two to eight patients (Table 1). Within each analyzed antigen, only a subset of overlapping peptides was recognized, whereas most peptides were not recognized indicating that these subsets own preferred immunogenic characteristics to induce an immune response (Figure 6 and Supplementary Figure S4). Remarkably, some of these overlapping peptides were recognized by sera of more than one patient ("shared immune response"): in particular, 50–70% of treated patients shared response to immunogenic regions within survivin, stromelysin, c-myc, Histone H1.2, and G1/S-specific cyclin-D1, indicating their comprehensive immunogenic potential (Table 1). Furthermore, 20–40% of treated patients shared immune response to peptides within G2/mitotic-specific cyclin-B1, Met, PRAME, telomerase, p53, apolipoprotein L1, and MAGE A1, whereas the antigens MUC1, NY-ESO-1, WT-1, Her-2/neu, and MAGE A3 did not show any overlapping peptides recognized by more than one patient. However, individual immune responses against peptides within these antigens could be detected confirming their immunogenic potential, i.e., 7 out of 10 patients developed antibodies against Her-2/neu, and 3 patients against NY-ESO-1 and MAGE A3.

DISCUSSION

A variety of cancer vaccines are currently evaluated in clinical trials, and sipuleucel-T (DC-based) is the first tumor vaccine[25] approved by the US Food and Drug Administration (2010) and European Medicines Agency (2013). Here, the development of a new kind of cell-based tumor vaccine, MGN1601 consisting of fourfold gene-modified vaccine cells (originally derived from tumor material) in combination with the TLR-9 agonist dSLIM, is described as well as its translation from use in mice to its application in patients.

For the proof-of-concept studies, a syngeneic vaccination model was chosen. Use of the murine homologue of MGN1601 in a syngeneic murine setting unambiguously showed repression of tumor growth: tumor growth was completely repressed if the vaccine was applied in a prophylactic setting adding up in a survival rate of 100% (Figure 1b). Thereafter, in the therapeutic setting, efficacy of experimental variants of the vaccine was compared to evaluate the therapeutic concept. Therapeutic application of the murine vaccine impressively reduced tumor growth in mice (Figure 1d). It was demonstrated that the combination of the vaccine cells, their fourfold gene modification, and the TLR-9 agonist dSLIM are necessary to achieve effective antitumor properties probably by synergistic effects, e.g., by linking of innate and adaptive immunity.[6,26,27] For syngeneic models, it is known that gene modification of vaccine cells improves survival of mice in melanoma models (B16F10 and K1735-M2).[28] Due to the complete match of MHC molecules of vaccine cells with the tumor cells, the immunological stimulus is weaker compared to allogeneic setting, but it is intrinsically assured that the TAA expressed by the growing tumor are also expressed by the vaccine cells. The shared expression of TAA by vaccine cells and tumor cells is supposed to be crucial for the induction of a protective immune response. This

Table 1 Analysis of patients' sera by peptide array: identification of shared immune responses[a]

Antigen information				*Immune response information*			
				Individual immune response	*Shared immune response*		
Antigen	*Gene*	*mRNA expression (log_2-based) in B25MOL*	*Peptides/ AG (N)*	*Patients (N)*	*Patients (N)*	*Peptide number*	*Patient ID*
Survivin	*BIRC5*	9.0	71	7	7	36–40	1, 4, 8, 9, 16, 17, 19
Stromelysin 3	*MMP11*	8.0	244	7	6	98–100	1, 4, 8, 9, 10, 16
					3	155–156	1, 8, 17
					3	178–183	8, 9, 17
					3	223–225	4, 8, 9
c-myc	*MYC*	10.2	219	7	5	12–19	4, 8, 9, 10, 16
					5	130–135	1, 8, 9, 16, 17
Histon H1.2	*HIST1H1C*	9.4	106	6	4	12–18	1, 9, 10, 14
					5	69–108	1, 9, 10, 16, 19
G1/S-specific cyclin-D1	*CCND1*	11.1	148	5	2	38–40	8, 17
					5	138–144	8, 9, 10, 16, 17
G2/mitotic-specific cyclin-B1	*CCNB1*	9.6	217	8	4	51–57	1, 16, 17, 19
Met	*MET*	12.6	692	8	2	81–84	4,17
					2	147–151	10, 19
					2	554	3, 10
					3	611	8, 17, 19
					2	650	4, 10
PRAME	*PRAME*	7.8	262	5	2	27–29	4, 9
					3	102–104	4, 10, 14
Telomerase	*TERT*	8.1	566	8	3	221–223	4, 8, 17
					2	435–437	9, 10
p53	*TP53*	9.6	197	5	2	42–47	8, 16
					2	180–181	1, 3
Apolipoprotein L1	*APOL1*	10.2	199	3	2	161–163	9, 10
MAGE A1	*MAGEA1*	6.6	155	3	2	132–133	9, 17
Her-2/neu	*ERBB2*	8.7	627	7	0	—	—
Mucin 1	*MUC1*	8.1	236	5	0	—	—
NY-ESO-1	*CTAG1A*	8.0	91	3	0	—	—
MAGE A3	*MAGEA3*	6.8	164	3	0	—	—
WT-1	*WT1*	7.8	224	2	0	—	—

PP, per protocol.

[a]Sera of all PP-treated patients ($N = 10$) from the ASET study were collected before the first vaccination and after eight vaccinations (week 12) and analyzed for binding intensity to 17 antigens. Each antigen was represented by successive 15-mer peptides with an overlap of 13 amino acids. Each peptide was spotted in duplicate to the array. Immune response to peptides was identified when three criteria were fulfilled: high binding intensity (>1,000) of the serum combined with a greater than twofold increase of binding signal compared to the preimmune sera and binding intensity to overlapping peptides (with at least binding signal of 500). Number of patients with individual immune response to peptides of the respective antigen is given. Additionally, the number of patients with shared immune responses is given (more than one serum binds to the same set of overlapping peptides) for each respective set of overlapping peptides within the TAA. The "spotting numbers" of these peptides are given as well as the respective patient ID.

aspect is not assured by an allogeneic murine vaccination setting and requires the careful selection of a suitable cell line. Therefore, in the human situation, the expression of TAA in B25MOL cells was a critical aspect for their selection as basis of the vaccine.

MGN1601 was designed to mainly induce an especially Th1-based immune response against tumor cells. IL-7, a potent growth factor for T-cells, should support activation, maturation, and proliferation of T cells involved in MGN1601 response, whereas presence

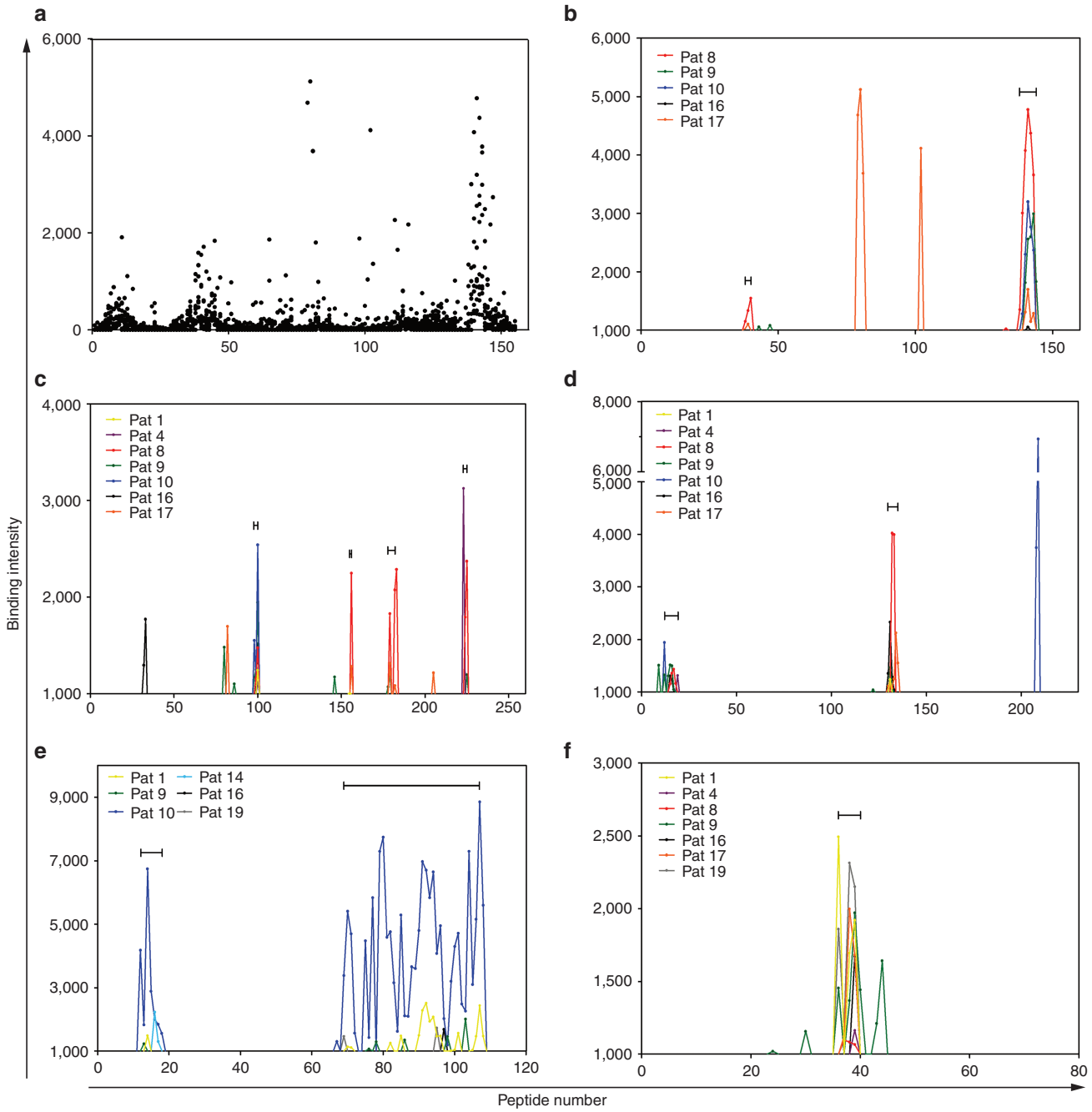

Figure 6 Induction of antibodies against TAA by vaccination with MGN1601 in mRCC patients. Sera of all PP-treated patients ($N = 10$) from the ASET study were collected before the first vaccination and after eight vaccinations (week 12) and analyzed for binding intensity to 17 antigens (see also Supplementary Figures S3 and S4). Each antigen was represented by successive 15-mer peptides with an overlap of 13 amino acids. Each peptide was spotted in duplicate to the array. (**a**) Binding intensities (sera of all 10 patients) against all peptides of cyclin D1 (without any criterion for immune response). (**b**–**f**) Binding intensities of sera to peptides of specified TAA are depicted when three immune response criteria were fulfilled: high binding intensity (>1,000) of the serum combined with a greater thantwofold increase of binding signal compared to the preimmune sera and binding intensity in serum to overlapping peptides (with at least binding signal of 500). "⊢" indicates shared immunogenic regions. (**b**) cyclin D1, (**c**) stromelysin 3, (**d**) c-myc, (**e**) histone H1.2, and (**f**) survivin. PP, per protocol.

of GM-CSF supports recruitment and maturation of APC, especially DC, and enhances cross-presentation as well as cross-priming.[29–31] Indeed, presence of T cells and APC at the sites of injection of the vaccine was highly increased in mice (Figure 2a and Supplementary Figure S2). Vaccine cells act themselves like a kind of APC especially due to their expression of CD80. CD80 is described to improve immunogenicity of co-expressed TAA, thereby supporting T-cell response.[32] In fact, tumor-specific T cells are activated, as evident by increased specific cytotoxicity of spleen cells in the mouse model (Figure 2c) with an at least 4-week lasting effect (recovery group).

As discussed above the expression of TAA in B25MOL cells was a critical aspect for their selection. Many of these TAA are known to own immunogenic potential. Expression of TAA at protein level on the cell surface was roughly maintained on vaccine cells when compared to B25MOL source cells (Figure 5b). This collection of endogenously expressed TAA is an indispensable signal for T-cell activation,

whereas gene modification of B25MOL cells with the co-stimulatory molecules CD80 and CD154 provide their activation signals (CD154 via activation of APC). As shown in Figure 4a,c, about 50% of vaccine cells express both co-stimulatory molecules. Expression of cytokines is not necessarily required to happen in the same cell, as the cytokines are secreted. It is important to note that all four proteins are expressed and—in case of cytokines—secreted, despite irradiation of gene-modified cells (Figure 4b). *In vivo*, the concentration of cytokines at the application site of mice increased for 8 hours and decreases thereafter, most probably due to degradation of cytokines, cytokine consumption, and their drainage from the application site to other body compartments like blood or lymph (Figure 4d). High local concentration of allogeneic tumor cells expressing their TAA (Supplementary Table S2) and the co-stimulatory molecules CD80 and CD154 in the presence of the cytokines IL-7 and GM-CSF as well as the TLR-9 agonist dSLIM is expected to result in an immune-responsive microenvironment at the site of vaccination. High presence of $CD4^+$ and $CD8^+$ T cells as well as $CD86^+$ APC was confirmed by immunohistochemistry of the injection sites.

CD4-based humoral immune response was assessed in vaccinated patients for a variety of TAA (about a dozen thereof are described by Cheever *et al*.[33]) using peptide arrays. Most strikingly, overlapping peptides were identified that had induced an immune response shared by up to 7 (out of 10) patients (Table 1), indicating a shared immune response to comprehensive immunogenic regions within some TAA. Among these is G1/S-specific cyclin-D1, a regulator of cyclin-dependent kinase which is overexpressed in many types of cancer and used as experimental target for tumor therapies.[34] In the ASET study, 5 out of 10 patients developed antibodies reactive to the same or adjacent peptides within cyclin D1 ("shared immunogenic region"). These data are consistent with others that identified several peptides from cyclin D1 as T-cell epitopes.[35] Another promising molecule for immunotherapeutic approach is survivin,[36] an inhibitor of apoptosis. Currently there are several trials going on with survivin vaccines.[37] In the ASET study, 7 out of 10 of PP-vaccinated patients developed antibodies against a dedicated region within survivin confirming the therapeutic potential of this molecule. Another TAA expressed in B25MOL cells is Met (Supplementary Table S2 and Figure 3b), the receptor for hepatocyte growth factor receptor. High protein expression of Met correlates with poor prognosis in RCC, and about 66% of RCC tumor material is positive for Met.[38] The mRNA of *MET* is upregulated in clear cell RCC compared to normal kidney[39] which was confirmed for B25MOL cells. Vaccination of patients with MGN1601 resulted in the elicitation of a shared immune response to Met of three patients to comprehensive immunogenic regions. However, individual Met-specific antibodies to overlapping peptides were detected in 7 out of 10 tested ASET patients (Table 1). These results are in line with other attempts using Met and its downstream signaling pathway as target for treatment of RCC.[40] Furthermore, the human epidermal growth factor receptor 2, Her-2/neu, is an important target in mammary carcinoma[41,42] and the focus of numerous active immunotherapy approaches.[43] More than 30% of RCC tumors express Her-2/neu,[44] and Her-2/neu is expressed at medium intensity on B25MOL cell surface, whereas *ERBB2*-mRNA is highly expressed. Vaccination of patients with MGN1601 induced antibodies that recognized peptides of Her-2/neu in 7 out of 10 analyzed sera (individual immune response), while comprehensive immunogenic regions were not detected. A further well-known candidate for immunotherapy is MUC1 (CD227).[45,46] It is expressed on RCC tumor material as well as on B25MOL cells (with medium intensity). A moderate discrepancy between mRNA data and fluorescence-activated cell sorting data in the vaccine cells may be explained by intracellular localization or shedding of MUC1. Five out of 10 patients developed individual MUC1-specific antibodies after vaccination with MGN1601 although not to comprehensive immunogenic regions. Other cancer-related antigens with known immunogenic potential like CD326 (EpCAM),[47] CD146 (MCAM or MUC18),[48] CD44, CD105 (endoglin), and CD151[49] are expressed at mRNA and protein levels in reasonable or high amounts in B25MOL cells. The evaluation of immune response to those TAA remains to further studies. The observed increase in antibody titer after vaccination with MGN1601 is most probably due to either enhanced B-cell activation or activation of memory B cells by the vaccine. The finding of a shared immune response to the same peptides highlights the immunogenicity of the respective peptides. Nevertheless, it cannot be excluded that changes in the tumor mass or natural fluctuations may influence the titer of antibodies and contribute to this effect. Number of evaluated sera is too low to allow correlation of antibody titer with survival. This will be the aim of further studies.

According to the antibody response in patients, vaccination of mice with the murine homologue generated high titers of antibodies responsive to lysate of Renca cells (Figure 2b). CD4-based humoral immune response is supported by the combined actions of CD154 and GM-CSF and in keeping with the ability of dSLIM to activate natural killer cells and B cells,[7] thus probably increasing the antitumor effect via antibody-dependent cellular cytotoxicity.

MGN1601 was further designed to support cross-presentation and cross-priming, a process described to be essential for success of tumor vaccines especially in a non–HLA-matched setting. Cross-presentation means the presentation of foreign antigens on HLA class I molecules by APC, especially DC, whereas cross-priming is the downstream activation of $CD8^+$ T cells to foreign antigens in lymph nodes. Besides the implication of GM-CSF[31] and CD154, cross-priming is further supported by the use of irradiated vaccine cells. Irradiation induces apoptosis, and apoptotic cells own an increased potential for cross-presentation.[50] Finally, TLR-9 agonists are shown to enhance cross-priming.[27,51] Combined effects of these aspects should result in efficient cross-priming by MGN1601. Whereas combination of a cell-based vaccination strategy with CpG-ODN as TLR-9 agonists had been shown to increase efficacy of prophylactic vaccination regimens, their efficacy in therapeutic vaccination studies remains a matter of controversy.[19,52–54]

Recently, clinical guidelines for immune-related response criteria were developed especially for immunotherapeutic studies of cancer due to the delayed response of immunologic treatment approaches.[55,56] In fact, a delayed response to the vaccine was observed in mice, illustrated by the kinetic of tumor growth suppression (Figure 1d). Additionally, patients with mRCC showed a significant benefit in overall survival only when receiving MGN1601 therapy over 12 weeks (eight applications) indicating that the improved cellular and humoral immune function during MGN1601 treatment requires time to be translated into clinical responses.

MGN1601 is the first tumor vaccine applied to humans using fourfold gene-modified vaccine cells in combination with a TLR-9 agonist and owns high potential to treat RCC. This vaccine may also be adapted for therapies of different tumor entities.

MATERIALS AND METHODS

Manufacturing of MGN1601

B25MOL cells were harvested and transiently gene modified with MIDGE vectors, encoding the human forms of CD80, CD154, IL-7, and GM-CSF via electroporation. Gene-modified cells were combined to a homogenous

batch of about 2×10^9 cells, gamma irradiated with 100 Gy, and stored in aliquots in the vaporous phase of liquid nitrogen. Gene-modified and irradiated cells after thawing and washing are called "vaccine cells." One dose of MGN1601 comprised 1×10^7 vaccine cells (in a syringe) supplemented with 5 mg dSLIM and was applied to patients within 24 hours after final preparation. Quality control data show a cell number of $0.9\times10^7 \pm 0.7\times10^6$ and a viability of 94.5% (± 1.5) ($N > 100$). Expression of CD80 and CD154 was analyzed by flow cytometry, whereas expression of cytokines was quantified by specific enzyme-linked immunosorbent assay in the cell culture supernatant. For more details, see Supplementary Materials and Methods.

Animal studies

For cytokine quantification at the injection site, NMRI mice were s.c. vaccinated (1×10^7 vaccine cells + 5 mg dSLIM) once at two different sides with the murine homologue and sacrificed after the indicated time points.

Antibody production and accumulation of immune cells at the injection site were evaluated by vaccinating NMRI mice (2×10^6 vaccine cells + 1 mg dSLIM) eight times with the murine homologue of MGN1601 at four sites once a week. Antibody titers were analyzed from blood samples. Accumulation of immune cells at the injection site was ranked on frozen tissue sections stained for T cells and APC by immunohistochemistry. For more details, see Supplementary Materials and Methods.

For determination of Renca-specific cytotoxic activity of spleen cells and assessment of subchronic toxicity, NMR mice received one dose of vaccine (low dose: 2×10^6 vaccine cells + 1 mg dSLIM; high dose: 1×10^7 vaccine cells + 5 mg dSLIM) or PBS as vehicle at four sites once a week for 13 consecutive weeks (six mice per sex and group). One week (5 weeks for recovery group) after the last application, mice were sacrificed, spleen cells were isolated, and co-cultured for 12 hours with Renca cells in the presence of IL-2 (Gibco/Invitrogen, Karlsruhe, Germany, PMC0023). Thereafter, re-stimulated spleen cells were mixed with fresh Renca cells that were analyzed 6 hours later for viability. For more details, see Supplementary Materials and Methods.

Balb/c received once weekly a dose of the murine vaccine (2×10^6 vaccine cells + 50 µg dSLIM) for prophylactic vaccination. After four applications, tumor was inoculated by application of 5×10^5 native Renca cells. For more details, see Supplementary Materials and Methods.

Therapeutic vaccination of Balb/c mice was performed using tumor inoculation with Renca cells before treatment with the murine vaccine started with one dose per treatment and week for 4 weeks. Experimental variants of the vaccine were "Tf Renca" (gene-modified Renca cells without dSLIM), "Renca" (irradiated Renca cells without dSLIM), and "dSLIM" (dSLIM as single component). PBS was used as control. For more details, see Supplementary Materials and Methods.

Single- and repeated-dose (five times) testing of MGN1601 in rats (heterologous setting) was performed by application of up to 1.15×10^7 vaccine cells (+ 1,150 µg dSLIM) by the subcutaneous route (single dose) or up to 2.3×10^6 vaccine cells (+ 230 µg dSLIM) per application every 5 days by intradermal route.

Analysis of immune response to TAA by peptide array

A phase 1/2 clinical ASET trial (ClinicalTrials.gov: NCT01265368; EudraCT No.: 2009-016853-16) was performed[24] in accordance with the declaration of Helsinki, and the international conference on harmonization Guideline for Good Clinical Practice. All patients gave written, informed consent prior to participation. MGN1601 was administered intradermally eight times over 12 weeks. Sera of patients treated per protocol were analyzed by peptide array. Seventeen TAA were selected (UniProt Entry number are given in brackets): MET (P08581), apolipoprotein L1 (O14791), G1/S-specific cyclin-D1 (P24385), telomerase (O14746), MUC1 (P15941), survivin (Q5RAH9), Myc (P01106), p53 (Q2XN98), histone H1.2 (P16403), NY-ESO-1 (P78358), Her-2/neu (P04626), MMP11 (P24347), WT-1 (P19544), G2/mitotic-specific cyclin B1 (P14635), PRAME (P78395), MAGE A3 (P43357), and MAGE A1 (P43355). Sequences were transformed into 15-mer peptides with a peptide–peptide overlap of 13 amino acids resulting in 4,399 different peptides that were spotted in duplicate onto the array. As control, the peptide microarrays were further framed by Flag and Hemagglutinin peptides on top and bottom ($N = 68$, each Flag and HA). Corresponding binding intensities of preimmune sera gained before the first application of MGN1601 and sera gained after the eighth application (week 12) of MGN1601 during the ASET study were analyzed for all 10 PP-treated patients. Signal intensities are given in arbitrary units. For identification of an (individual) immune response to a respective peptide, three aspects have to be met: signal intensity of sera > 1,000 and ratio (sera/preimmune sera) >2 plus at least one adjacent peptide showing binding intensity of >500. For identification of a shared immune response, sera of at least two patients have to meet the above-mentioned criteria for the same or adjacent peptides. For more details, see Supplementary Materials and Methods.

Ethics

All studies involving animals (mice) were performed in accordance with good laboratory practice regulations of the ethics committee.

CONFLICT OF INTEREST

B.V., M.S., K.H., K.K., and M.S. are employees of Mologen AG. B.W. is founder and shareholder of Mologen AG, a member of their scientific advisory board and receives funding for the Foundation Institute Molecular Biology and Bioinformatics from Mologen AG. dSLIM and MIDGE are registered trademarks. MGN1601 is property of Mologen AG.

ACKNOWLEDGMENTS

We thank Viktor Grünwald, Stefan Weikert, Ingo Schmidt-Wolf, Stefan Hauser, and Ahmed Maghel for conducting the ASET trial and providing preliminary clinical data.

REFERENCES

1. Ogi, C and Aruga, A (2013). Immunological monitoring of anticancer vaccines in clinical trials. *Oncoimmunology* **2**: e26012.
2. Vujanovic, L and Butterfield, LH (2007). Melanoma cancer vaccines and anti-tumor T cell responses. *J Cell Biochem* **102**: 301–310.
3. Endmann, A, Baden, M, Weisermann, E, Kapp, K, Schroff, M, Kleuss, C *et al.* (2010). Immune response induced by a linear DNA vector: influence of dose, formulation and route of injection. *Vaccine* **28**: 3642–3649.
4. Schakowski, F, Gorschlüter, M, Junghans, C, Schroff, M, Buttgereit, P, Ziske, C *et al.* (2001). A novel minimal-size vector (MIDGE) improves transgene expression in colon carcinoma cells and avoids transfection of undesired DNA. *Mol Ther* **3**: 793–800.
5. Schmidt, M, Volz, B, and Wittig, B (2005). MIDGE vectors and dSLIM immunomodulators: DNA-based molecules for gene therapeutic strategies. In: Knäblein, J, Müller, RH (eds). *Modern Biopharmaceutical*. WILEY-VCH Verlag GmbH&Co. KGaA: Weinheim. pp. 1–29.
6. Bode, C, Zhao, G, Steinhagen, F, Kinjo, T and Klinman, DM (2011). CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* **10**: 499–511.
7. Kapp, K, Kleuss, C, Schroff, M and Wittig, B (2014). Genuine immunomodulation with dSLIM. *Mol Ther Nucleic Acids* **3**: e170.
8. Schmidt, M, Anton, K, Nordhaus, C, Junghans, C, Wittig, B and Worm, M (2006). Cytokine and Ig-production by CG-containing sequences with phosphorodiester backbone and dumbbell-shape. *Allergy* **61**: 56–63.
9. Schmidt, M, Hagner, N, Marco, A, König-Merediz, SA, Schroff, M and Wittig, B (2015). Design and structural requirements of the potent and safe TLR-9 agonistic immunomodulator MGN1703. *Nucleic Acid Ther* **25**: 130–140.
10. Schmoll, HJ, Wittig, B, Arnold, D, Riera-Knorrenschild, J, Nitsche, D, Kroening, H *et al.* (2014). Maintenance treatment with the immunomodulator MGN1703, a Toll-like receptor 9 (TLR9) agonist, in patients with metastatic colorectal carcinoma and disease control after chemotherapy: a randomised, double-blind, placebo-controlled trial. *J Cancer Res Clin Oncol* **140**: 1615–1624.
11. Weihrauch, MR, Richly, H, von Bergwelt-Baildon, MS, Becker, HJ, Schmidt, M, Hacker, UT *et al.* (2015). Phase I clinical study of the toll-like receptor 9 agonist MGN1703 in patients with metastatic solid tumours. *Eur J Cancer* **51**: 146–156.
12. Biswas, S and Eisen, T (2009). Immunotherapeutic strategies in kidney cancer–when TKIs are not enough. *Nat Rev Clin Oncol* **6**: 478–487.
13. Pal, SK, Hu, A and Figlin, RA (2013). A new age for vaccine therapy in renal cell carcinoma. *Cancer J* **19**: 365–370.
14. Micka, B, Trojaneck, B, Niemitz, S, Lefterova, P, Kruopis, S, Huhn, D *et al.* (2000). Comparison of non-viral transfection methods in melanoma cell primary cultures. *Cytokine* **12**: 828–833.
15. Möller, P, Sun, Y, Dorbic, T, Alijagic, S, Makki, A, Jurgovsky, K *et al.* (1998). Vaccination with IL-7 gene-modified autologous melanoma cells can enhance the anti-melanoma lytic activity in peripheral blood of patients with a good clinical performance status: a clinical phase I study. *Br J Cancer* **77**: 1907–1916.
16. Sun, Y, Jurgovsky, K, Möller, P, Alijagic, S, Dorbic, T, Georgieva, J *et al.* (1998). Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and a first clinical phase I study. *Gene Ther* **5**: 481–490.
17. Wittig, B, Märten, A, Dorbic, T, Weineck, S, Min, H, Niemitz, S *et al.* (2001). Therapeutic vaccination against metastatic carcinoma by expression-modulated and immunomodified autologous tumor cells: a first clinical phase I/II trial. *Hum Gene Ther* **12**: 267–278.

18. Parmiani, G, Pilla, L, Maccalli, C and Russo, V (2011). Autologous versus allogeneic cell-based vaccines? *Cancer J* **17**: 331–336.
19. Köchling, J, König-Merediz, SA, Stripecke, R, Buchwald, D, Korte, A, Von Einsiedel, HG *et al.* (2003). Protection of mice against Philadelphia chromosome-positive acute lymphoblastic leukemia by cell-based vaccination using nonviral, minimalistic expression vectors and immunomodulatory oligonucleotides. *Clin Cancer Res* **9**: 3142–3149.
20. Liu, KJ, Lu, LF, Cheng, HT, Hung, YM, Shiou, SR, Whang-Peng, J *et al.* (2004). Concurrent delivery of tumor antigens and activation signals to dendritic cells by irradiated CD40 ligand-transfected tumor cells resulted in efficient activation of specific CD8+ T cells. *Cancer Gene Ther* **11**: 135–147.
21. Loskog, A and Tötterman, TH (2007). CD40L - a multipotent molecule for tumor therapy. *Endocr Metab Immune Disord Drug Targets* **7**: 23–28.
22. Julian, J, Dharmaraj, N and Carson, DD (2009). MUC1 is a substrate for gamma-secretase. *J Cell Biochem* **108**: 802–815.
23. Lillehoj, EP, Han, F and Kim, KC (2003). Mutagenesis of a Gly-Ser cleavage site in MUC1 inhibits ectodomain shedding. *Biochem Biophys Res Commun* **307**: 743–749.
24. Grünwald V, Weikert S, Schmidt-Wolf IGH, Hauser S, Magheli A, Schroff M, Schmidt M, Wittig B (2014). ASET study: Long-term follow-up data of patients with metastatic renal cell carcinoma treated with MGN1601. *J Clin Oncol* **32**: LBA399.
25. Cheever, MA and Higano, CS (2011). PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. *Clin Cancer Res* **17**: 3520–3526.
26. Krieg, AM (2008). Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. *Oncogene* **27**: 161–167.
27. Lande, R and Gilliet, M (2010). Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. *Ann NY Acad Sci* **1183**: 89–103.
28. Labarthe, MC, Halanek, N, Birchall, L, Russell, N, Desel, C, Todryk, S *et al.* (2006). The biological effects of syngeneic and allogeneic cytokine-expressing prophylactic whole cell vaccines and the influence of irradiation in a murine melanoma model. *Cancer Immunol Immunother* **55**: 277–288.
29. Jinushi, M, Hodi, FS and Dranoff, G (2008). Enhancing the clinical activity of granulocyte-macrophage colony-stimulating factor-secreting tumor cell vaccines. *Immunol Rev* **222**: 287–298.
30. van der Most, RG, Currie, A, Robinson, BW and Lake, RA (2006). Cranking the immunologic engine with chemotherapy: using context to drive tumor antigen cross-presentation towards useful antitumor immunity. *Cancer Res* **66**: 601–604.
31. Zhan, Y, Carrington, EM, van Nieuwenhuijze, A, Bedoui, S, Seah, S, Xu, Y *et al.* (2011). GM-CSF increases cross-presentation and CD103 expression by mouse $CD8^+$ spleen dendritic cells. *Eur J Immunol* **41**: 2585–2595.
32. Johnson, BD, Gershan, JA, Natalia, N, Zujewski, H, Weber, JJ, Yan, X *et al.* (2005). Neuroblastoma cells transiently transfected to simultaneously express the co-stimulatory molecules CD54, CD80, CD86, and CD137L generate antitumor immunity in mice. *J Immunother* **28**: 449–460.
33. Cheever, MA, Allison, JP, Ferris, AS, Finn, OJ, Hastings, BM, Hecht, TT *et al.* (2009). The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* **15**: 5323–5337.
34. Musgrove, EA, Caldon, CE, Barraclough, J, Stone, A and Sutherland, RL (2011). Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* **11**: 558–572.
35. Neumann, A, Hörzer, H, Hillen, N, Klingel, K, Schmid-Horch, B, Bühring, HJ *et al.* (2013). Identification of HLA ligands and T-cell epitopes for immunotherapy of lung cancer. *Cancer Immunol Immunother* **62**: 1485–1497.
36. Mobahat, M, Narendran, A and Riabowol, K (2014). Survivin as a preferential target for cancer therapy. *Int J Mol Sci* **15**: 2494–2516.
37. Kelly, RJ, Lopez-Chavez, A, Citrin, D, Janik, JE and Morris, JC (2011). Impacting tumor cell-fate by targeting the inhibitor of apoptosis protein survivin. *Mol Cancer* **10**: 35.
38. Miyata, Y, Kanetake, H and Kanda, S (2006). Presence of phosphorylated hepatocyte growth factor receptor/c-Met is associated with tumor progression and survival in patients with conventional renal cell carcinoma. *Clin Cancer Res* **12**: 4876–4881.
39. Behbahani, TE, Thierse, C, Baumann, C, Holl, D, Bastian, PJ, von Ruecker, A *et al.* (2012). Tyrosine kinase expression profile in clear cell renal cell carcinoma. *World J Urol* **30**: 559–565.
40. Harshman, LC and Choueiri, TK (2013). Targeting the hepatocyte growth factor/c-Met signaling pathway in renal cell carcinoma. *Cancer J* **19**: 316–323.
41. Ladjemi, MZ, Jacot, W, Chardès, T, Pèlegrin, A and Navarro-Teulon, I (2010). Anti-HER2 vaccines: new prospects for breast cancer therapy. *Cancer Immunol Immunother* **59**: 1295–1312.
42. Wiedermann, U, Davis, AB and Zielinski, CC (2013). Vaccination for the prevention and treatment of breast cancer with special focus on Her-2/neu peptide vaccines. *Breast Cancer Res Treat* **138**: 1–12.
43. Milani, A, Sangiolo, D, Montemurro, F, Aglietta, M and Valabrega, G (2013). Active immunotherapy in HER2 overexpressing breast cancer: current status and future perspectives. *Ann Oncol* **24**: 1740–1748.
44. Seliger, B, Rongcun, Y, Atkins, D, Hammers, S, Huber, C, Störkel, S *et al.* (2000). HER-2/neu is expressed in human renal cell carcinoma at heterogeneous levels independently of tumor grading and staging and can be recognized by HLA-A2.1-restricted cytotoxic T lymphocytes. *Int J Cancer* **87**: 349–359.
45. Oudard, S, Rixe, O, Beuselinck, B, Linassier, C, Banu, E, Machiels, JP *et al.* (2011). A phase II study of the cancer vaccine TG4010 alone and in combination with cytokines in patients with metastatic renal clear-cell carcinoma: clinical and immunological findings. *Cancer Immunol Immunother* **60**: 261–271.
46. Aubert, S, Fauquette, V, Hémon, B, Lepoivre, R, Briez, N, Bernard, D *et al.* (2009). MUC1, a new hypoxia inducible factor target gene, is an actor in clear renal cell carcinoma tumor progression. *Cancer Res* **69**: 5707–5715.
47. Armeanu-Ebinger, S, Hoh, A, Wenz, J and Fuchs, J (2013). Targeting EpCAM (CD326) for immunotherapy in hepatoblastoma. *Oncoimmunology* **2**: e22620.
48. Leslie, MC, Zhao, YJ, Lachman, LB, Hwu, P, Wu, GJ and Bar-Eli, M (2007). Immunization against MUC18/MCAM, a novel antigen that drives melanoma invasion and metastasis. *Gene Ther* **14**: 316–323.
49. Stickel, JS, Weinzierl, AO, Hillen, N, Drews, O, Schuler, MM, Hennenlotter, J *et al.* (2009). HLA ligand profiles of primary renal cell carcinoma maintained in metastases. *Cancer Immunol Immunother* **58**: 1407–1417.
50. Nowak, AK, Lake, RA, Marzo, AL, Scott, B, Heath, WR, Collins, EJ *et al.* (2003). Induction of tumor cell apoptosis *in vivo* increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J Immunol* **170**: 4905–4913.
51. de Brito, C, Tomkowiak, M, Ghittoni, R, Caux, C, Leverrier, Y and Marvel, J (2011). CpG promotes cross-presentation of dead cell-associated antigens by pre-$CD8\alpha$+ dendritic cells [corrected]. *J Immunol* **186**: 1503–1511.
52. Sandler, AD, Chihara, H, Kobayashi, G, Zhu, X, Miller, MA, Scott, DL *et al.* (2003). CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. *Cancer Res* **63**: 394–399.
53. Shirota, H and Klinman, DM (2011). CpG-conjugated apoptotic tumor cells elicit potent tumor-specific immunity. *Cancer Immunol Immunother* **60**: 659–669.
54. Switaj, T, Jalili, A, Jakubowska, AB, Drela, N, Stoksik, M, Nowis, D *et al.* (2004). CpG immunostimulatory oligodeoxynucleotide 1826 enhances antitumor effect of interleukin 12 gene-modified tumor vaccine in a melanoma model in mice. *Clin Cancer Res* **10**: 4165–4175.
55. Fox, BA, Schendel, DJ, Butterfield, LH, Aamdal, S, Allison, JP, Ascierto, PA *et al.* (2011). Defining the critical hurdles in cancer immunotherapy. *J Transl Med* **9**: 214.
56. Hoos, A, Eggermont, AM, Janetzki, S, Hodi, FS, Ibrahim, R, Anderson, A *et al.* (2010). Improved endpoints for cancer immunotherapy trials. *J Natl Cancer Inst* **102**: 1388–1397.

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