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MAGE-A antigens as targets for cancer immunotherapy

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ABSTRACT

Targeted anti-cancer therapies aim at reducing side effects while retaining their anti-cancer efficacy. Immunotherapies e.g. monoclonal antibodies, adoptive T cell therapy and cancer vaccines are used to combat cancer, but the number of available cancer specific targets is limited and new approaches are needed to generate more effective and patient tailored treatments. Unique cancer intracellular epitopes can be presented on the cell surface by MHC class I molecules, which can function as epitopes for targeted therapies. The intracellular MAGE proteins belong to a sub-class of Cancer Testis (CT) antigens which are expressed in germline cells and a wide variety of tumors of different histological origin. Evidence has emerged that their expression is linked to pro-tumorigenic activities like increased cell motility, resisting cell death, and tumor promoting inflammation. Intracellular MAGE proteins are processed by the proteasome and their peptides are presented by MHC class I molecules on the cell surface of cancer cells thereby making them ideal cancer specific antigens. Here we review the previous and ongoing (pre-) clinical studies on the use of surface expressed MAGE antigens for their employment in targeted anti-cancer therapies. We present and analyze study outcomes and discuss possible future directions and improvements for MAGE directed anti-cancer immunotherapies.

Introduction

Currently, most cancer patients receive a combination of surgical-, chemical- and radiation based therapies. The choice of the applied treatment depends on the type of tumor and progression state. Most of the available anticancer drugs do not have a highly tumor specific mode of action, which limits their therapeutic potential and may result in severe side effects [1]. The introduction of monoclonal antibodies (mAb), adoptive T cell therapy and therapeutic vaccines for cancer treatment has been a great step in bringing us closer towards personalized and more tumor specific medicine. However, one of the major challenges, being the design of a therapy that is at the same time efficacious and trully cancer-specific, still remains unresolved. Tumor-associated biomarkers can be divided into two classes: tumor-associated antigens (when the antigen is present on both healthy and cancer cells) and tumor specific antigens (when the antigen is restricted exclusively to cancer cells e.g. mutated p53) [2]. The majority of mAbs currently approved by the US Food and Drug Administration (FDA) and undergoing evaluation in clinical trials target extracellular antigens, more rarely to soluble proteins, that belong to the tumor-associated antigens class [3,4]. These antigens represent haematopoietic differentiation antigens (e.g. CD20), glycoproteins expressed by solid tumors (e.g. EpCAM, CEA or CAIX), glycolipids (i.e. gangliosides), carbohydrates (i.e. Lewis Y antigen), stromal and extracellular matrix antigens (e.g. FAP), proteins involved in angiogenesis (e.g. VEGFR or integrins), receptors involved in growth and differentiation signalling (e.g. EGFR, HER2 or IGF1R) and recently immune checkpoint proteins expressed on T cells (e.g. PD-1 or CTLA-4) [5]. A full list of currently clinically approved antibodies and their targets is presented in Table 1.

In order to ensure safety and efficacy of the therapy the ideal immunotherapeutic target should posses several characteristics: it should be stably expressed by cancer cells and exclusively accessible on cancer cells. Its possible shedding to the circulation should be minimal in order to avoid binding of targeting molecules that could result in decreased efficacy of the treatment. Depending on the therapeutic format, the target should either internalize slowly – desirable when for instance a certain antibody dependent effector function is needed, or on the contrary rapidly internalize, which is desirable when aiming at receptor downregulation or delivering toxic agents. Due to the fact that in cancer every single treatment becomes less effective as over time the cancer builds up an evasive response, the ideal target should be essential for cancer cell survival. Choice of target that would prevent tumor escape completely and thereby allow continuation of the treatment for a prolonged period of time is important.

Almost 30 years ago van der Bruggen et al. discovered the first member of a unique class of antigens by employing autologous typing

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Table 1
List of therapeutic antibodies approved by FDA and EMA until 1st January 2018.

No.	Drug name	Active ingredient	Target	Year of first approval by FDA	Year of first approval by EMA	Indication	Format
1	Rituxan	Rituximab	CD20	1997	1998	Non-Hodgkin's lymphoma	Human – mouse chimeric IgG1
2	Herceptin	Trastuzumab	HER2	1998	2000	Metastatic breast cancer, Gastric cancer	Humanized IgG1
3	Mylotarg[a]	Gemtuzumab ozogamicin	CD33	2000	–	Acute myeloid leukemia (AML)	Humanized IgG4 kappa, ADC
4	Campath	Alemtuzumab	CD52	2001	2001	B-cell chronic lymphocytic leukemia	Humanized IgG1
5	Zevalin[b]	Ibritumomab tiuxetan	CD20	2002	2004	Non-Hodgkin's lymphoma	Mouse IgG1
6	Bexxar[c]	Tositumomab, and I^{131} Tositumomab	CD20	2003	2003 (as orphan drug)	Non-Hodgkin's Lymphoma	Murine IgG2a lambda
7	Avastin	Bevacizumab	VEGF	2004	2005	Colorectal Cancer, Renal cell carcinoma	Humanized IgG1
8	Erbitux	Cetuximab	EGFR	2004	2004	Colorectal Cancer	Human – mouse chimeric IgG1
9	Vectibix	Panitumumab	EGFR	2006	2007	Colorectal cancer	Fully human IgG2
10	Removab	Catumaxomab	EpCAM & CD3	–	2009	Intraperitoneal treatment of patients with malignant ascites	BiTe, mouse IgG2a and rat IgG2b
11	Arzerra	Ofatumumab	CD20	2009	2010	Chronic lymphocytic leukemia	Fully human IgG1
12	Adcetris	Brentuximab vedotin	CD30	2011	2012	Hodgkin lymphoma and ALCL (systemic anaplastic large cell lymphoma	Human – mouse chimeric IgG1
13	Yervoy	Ipilimumab	Cytotoxic T lymphocyte-associated antigen 4	2011	2011	Late-stage (metastatic) melanoma	Fully human IgG1
14	Perjeta	Pertuzumab	HER2	2012	2013	HER2-positive late-stage (metastatic) breast cancer	Humanized IgG1
15	Kadcyla	Ado-trastuzumab emtansine	HER2	2013	2013	For patients with HER2-positive, late-stage (metastatic) breast cancer	Humanized IgG1, ADC
16	Gazyva	Obinutuzumab	CD20	2013	2014	Chronic lymphocytic leukemia (CLL)	Humanized IgG1
17	Xgeva	Denosumab	RANKL	2013	2011	Giant cell tumor of bone	Fully human IgG2
18	Cyramza	Ramucirumab	VEGFR2	2014	2014	Advanced stomach cancer or gastroesophageal junction adenocarcinoma	Fully human IgG1
19	Sylvant	Siltuximab	Il-6	2014	2014	Multicentric Castleman's disease (MCD)	Human – mouse chimeric IgG1
20	Keytruda	Pembrolizumab	PD-1 receptor	2014	2015	Advanced or unresectable melanoma, Metastatic non-small cell lung cancer, Head and neck squamous cell cancer	Humanized IgG4
21	Blincyto	Blinatumomab	CD19 & CD3	2014	2015	Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia (B-cell ALL)	BiTe
22	Opdivo	Nivolumab	PD-1 receptor	2014	2015	Unresectable or metastatic melanoma, Advanced renal cell carcinoma, Metastatic squamous non-small cell lung cancer, Classical Hodgkin lymphoma and Recurrent or metastatic squamous cell carcinoma of the head and neck	Fully human IgG4
23	Unituxin	Dinutuximab	Glycolipid GD2	2015	2015	Neuroblastoma	Human – mouse chimeric IgG1
24	Darzalex	Daratumumab	CD38	2015	2016	Multiple myeloma	Fully human IgG1
25	Portrazza	Necitumumab	EGFR	2015	2016	Metastatic squamous non-small cell lung cancer (NSCLC)	Fully human IgG1
26	Empliciti	Elotuzumab	SLAMF7 receptor	2015	2016	Multiple myeloma	Humanized IgG1
27	Tecentriq	Atezolizumab	PD-L1	2016	2017	Urothelial carcinoma	Fully human IgG1
28	Lartruvo	Olaratumab	PDGF-Rα	2016	2016	Certain types of soft tissue sarcoma	Fully human IgG1
29	Bavencio	Avelumab	PD-L1	2017	2017	Metastatic Merkel cell carcinoma	Fully human IgG1
30	Imfinzi	Durvalumab	PD-L1	2017	–	Locally advanced or metastatic urothelial carcinoma	Fully human IgG1
31	Besponsa	Inotuzumab ozogamicin	CD22	2017	2017	Relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL)	Humanized IgG4

[a] Available in the period of 2000–2010, in 2017 approved for adults with newly diagnosed CD33-positive acute myeloid leukemia (AML), and adults and children 2 years and older with relapsed or refractory CD33-positive AML.
[b] First radioimmunotherapy to receive FDA approval.
[c] Was withdrawn from the Community Register of designated Orphan Medicinal Products in March 2015.

with T-cell clones derived from a melanoma patient [6]. Shortly after this discovery more proteins sharing the same characteristics were identified [7–9]. Due to their expression pattern being restricted to germ cells of immuno-privileged testis and placenta, as well as a wide range of malignant cells, they were named cancer testis antigens (CT antigens). At first, members of CT family were identified based on serological analysis of recombinant tumor cDNA expression libraries (SEREX). However, recently their identification has been exclusively based on mRNA expression profiling in cancer and healthy cells [10]. Many similarities between germ and cancer cells led to the hypothesis that activation of normally silenced genes encoding the gametogenic programme in somatic cells is a driving force of tumorigenesis [11,12]. Expression of CT antigens in cancer cells was shown to result in their uncontrolled growth, resistance to cell death, potential to migrate, growth at distant sites (invasion and metastasis) and the ability to induce growth of new blood vessels (angiogenesis) [10,13]. Common characteristics shared by CT antigens include highly tissue-restricted expression profile, frequent mapping to chromosome X, existence as multigene families, induction of expression by hypomethylation and/or histone acetylation, and immunogenicity in cancer patients [13].

Proteins of the Melanoma Antigen Gene family (MAGE) were the first identified members of CT antigens. Over 60 proteins that belong to this family are further classified into two main categories based on the location of their genes [14]. Type I, which expression is limited to the X-chromosome, includes MAGE-A, -B and -C subfamilies, whereas type II, which is not restricted to the X-chromosome, includes MAGE-D, -E, -F, -G, -H, -L and Necdin [15]. All MAGE proteins are highly homologous and share a conservative MAGE Homology Domain (MHD). This approximately 200 amino acid long, helical region is suspected to be involved in protein-protein interactions [15,16]. MAGEs have been found to be broadly expressed in male germ line and placental cells, as well as in many tumor types, including melanoma, brain, lung, prostate, and breast, among others [17,18]. While clinical data points to a correlation between MAGE expression and poor prognosis, it has been lately suggested that expression of MAGE proteins is also associated with resistance to chemotherapy [10,16,19]. There is growing evidence supporting MAGE protein involvement in regulation of processes underlying cancer cell survival. MAGE proteins were shown to increase survival of cancer cells by direct interaction with p53 tumor supressor or indirectly by regulating activity of E3 RING ubiquitin ligases [10,20]. MAGE proteins were also shown to increase the metastatic potential of the cancer cells by enhancing cell motility and thereby their invasive potential [10].

Due to their intracellular expression MAGE proteins remain inaccessible targets until they undergo proteasomal degradation into short peptides in the cytoplasm. These peptides generated by the proteasome are then transported into endoplasmic reticulum where they are loaded onto the MHC class I molecules. Intracellularly processed MAGE-A derived peptides can be used as an immunotherapy target once present on the cell membrane in complex with MHC class I molecules. The high homology between MAGE-A proteins allows for identification of peptides that are shared by multiple members of the MAGE-A family. These multi-MAGE-A peptides presented by MHC molecules enable targeted therapy of tumors from different histological origin with highly heterogeneous expression of individual MAGE-A proteins in individual cells. Sequential targeting of different MAGE-A derived peptides in context of different MHC molecules creates the unique opportunity of efficacious treatment for a prolonged period of time.

In this review we will present results of completed studies which employed MAGE-A proteins as antigens in different immunotherapeutic approaches and discuss future directions in MAGE antigen-based immunotherapy.

Cancer immunotherapy directed against MAGE antigens

Already for some time it is clear that cancer cells can be immunogenic [21]. However, spontaneous immune responses against tumors are usually not sufficient to control or even reduce tumor growth [22]. Recent developments within the field of tumor immunology have boosted the rational design of cancer immunotherapeutic strategies. For instance, a better understanding of the phenomenon of immune evasion, which is seen as one of the hallmarks of cancer [23], has led to the development of a series of immunotherapeutic interventions (e.g. immune checkpoint inhibitors). One of the mechanisms of immune evasion is down-regulation of tumor antigens. Therefore, it would be beneficial to target tumor antigens that play a role in oncogenicity, such as MAGE antigens. In a study conducted by the National Cancer Institute in which 75 tumor antigens were prioritized according to several antigen characteristics (such as e.g. immunogenicity and oncogenicity), two CT antigens were listed in the top ten, namely MAGE-A3 (position 8) and NY-ESO-1 (position 10) [24]. While conventional therapies such as chemotherapy and radiation are effective for the majority of patients, the use of these modalities alone may be insufficient for patients with relapsed cancer [18]. Targeted immunotherapeutic cancer therapies like the use of monoclonal antibodies, re-directed T cells/NK cells or cancer vaccines show efficacy against tumor cells, while preserving healthy tissue. The restricted expression of CT antigens, together with their oncogenic potential, have directed research towards immunotherapeutic approaches in which CT antigens like MAGE have been used as a targets.

Targeting MAGE-A/MHC class I complexes using monoclonal antibodies or antibody fragments

Specific antigen recognition by the immune system is governed by T- and B cells. Through positive and negative selection processes in the thymus, T cells are trained to recognize antigens in form of linear peptides presented on the cell surface in complex with MHC molecules. Three dimentional antigen recognition by B cells is independent of MHC restriction. The development of phage display technology and advances in production of recombinant peptide HLA complexes folded in the native conformation enabled the development of antibodies recognizing antigens in a comparable manner as T cell receptors (TCR) present on the surface of T cells. Antibodies having these TCR-like specificities combine two main advantages of the immune system: the fine specificity of T cells and the biological and pharmacological properties of an antibody. Several antibodies or antibody fragments (Fabs) have been developed which are directed against human $CD8^+$ T-cell epitopes. Many groups have focused on raising mAbs recognizing tumor associated antigens like gp100, tyrosinase, or WT-1 [25–27]. Only three preclinical studies describe mAbs targeting peptides derived from MAGE-A proteins in context of HLA-A1 or HLA-A2.

In 2000 Chames et al. reported the discovery of a Fab fragment, further referred to as G8, binding exclusively to peptide derived from MAGE-A1 (amino acid 160–168; EADPTGHSY) in context of HLA-A1 [28]. The specificity of the G8 Fab was confirmed in multiple cellular assays, but it was considered a moderate affinity binder (250 nM). Therefore G8 was subjected to an affinity maturation process resulting in a high affinity clone, named Hyb3. The affinity of Hyb3 was 18-fold higher than that of parental G8, while specificity was retained [29].

In a recent study, Saeed and colleagues used G8 and Hyb3 reformatted into scFv for liposome targetting to MAGE-A1/HLA-A1 positive cells [30]. They have shown that both scFv Hyb3 and scFv G8 coupled to immunoliposomes remained functional in terms of binding to target cells, but they were also internalized. Importantly, scFv Hyb3, but not scFv G8 immunoliposomes demonstrated off-target binding to antigen-negative cells, both when tested using antigen-high B-cells, but also when using antigen-low melanoma cells. This undesired off-target binding was explained by the high affinity binding properties of scFv

Table 2
List of clinical trials registered at https://clinicaltrials.gov involving MAGE-A until 1st of January 2018.

No.	ClinicalTrials.gov Identifier	Phase	Status	Cancer type	MAGE-A	HLA type	Formulation	Sponsor
1	NCT02989064	I	Recruiting	Urinary Bladder Cancer, Head and Neck Cancer, Melanoma	MAGE-A10	HLA-A*02:01 and/or HLA-A*02:06	Autologous genetically modified MAGE A10^{c796}T cells	Adaptimmune
2	NCT03132922	I	Recruiting	Bladder Cancer, Melanoma, Head and Neck Cancer, Ovarian Cancer, Non-Small Cell Lung Cancer, Esophageal Cancer, Gastric Cancer	MAGE-A4	HLA-A*02	Autologous genetically modified MAGE-A4^{c1o32}T cells	Adaptimmune
3	NCT03139370	I	Recruiting	Advanced cancers	MAGE-A3/A6	HLA-DPB1*04:01	Autologous genetically modified MAGE-A3/A6 TCR transduced T cells (KITE-718)	Kite Pharma, Inc.
4	NCT02111850	I/II	Recruiting	Melanoma, Renal cancer, other metastatic cancers	MAGE-A3	HLA-DP0401 and HLA-DP0402	Autologous genetically modified MAGE-A3 TCR transduced T cells	National Cancer Institute (NCI)
5	NCT02153905	I/II	Recruiting	Metastatic Cancer, metastatic melanoma	MAGE-A3	HLA-A*01	Autologous genetically modified MAGE-A3 TCR transduced T cells	National Cancer Institute (NCI)
6	NCT02592577	I/II	Recruiting	Stage IIIb or Stage IV Non-Small Cell Lung Cancer (NSCLC)	MAGE-A10	HLA-A*02:01 and/or HLA-A*02:06	Autologous genetically modified MAGEA10^{c796}T cells	Adaptimmune
7	NCT01995708	I	Recruiting	Multiple Myeloma	MAGE-A3	–	Vaccine made with altered dendritic cells	Memorial Sloan Kettering Cancer Center
8	NCT02285816	I/II	Recruiting	Advanced/Metastatic Solid Tumours	MAGE-A3	–	Oncolytic Vaccine	Canadian Cancer Trials Group
9	NCT02096614	I	Recruiting	Solid tumors	MAGE-A4	HLA-A*24:02	MAGE-A4-specific TCR gene transduced T lymphocytes	Mie University
10	NCT02879760	I/II	Recruiting	Non-Small Cell Lung Cancer	MAGE-A3	–	Adenovirus Vaccine Expressing MAGE-A3	Turnstone Biologics, Inc.
11	NCT02203903	I	Recruiting	Relapsed/Refractory Hematopoietic Malignancies	MAGE-A3	–	Rapidly-generated multi-antigen-specific T lymphocytes	Catherine Bollard
12	NCT01333046	I	Recruiting	Hodgkin Lymphoma Non-Hodgkin Lymphoma Hodgkin Disease	MAGE-A4	–	Autologous TAA-specific cytotoxic T lymphocytes	Baylor College of Medicine
13	NCT01883518	I/II	Recruiting	Sarcoma Neoplasms, Connective and Soft Tissue	MAGE-A3	–	Autologous dendritic cell vaccine	Petrov Research Institute of Oncology
14	NCT02750995	I	Recruiting	Myelodysplastic Syndrome, Acute Myeloid Leukemia	MAGE-A3	–	Peptide vaccine	Inge Høgh Dufva
15	NCT00257738	I	Active, not recruiting	Squamous Cell Carcinoma of the Head and Neck	MAGE-A3	–	Peptide vaccine using Trojan complexes composed of CD4 and CD8 T-cell epitopes, connected by furin cleavable linkers	University of Maryland
16	NCT01245673	II	Active, not recruiting	Advanced Myeloma	MAGE-A3	–	Combination of immune system treatments (MAGE-A3 vaccine plus activated T-cells)	University of Pennsylvania
17	NCT01437605	II	Active, not recruiting	Melanoma	MAGE-A3	–	Vaccine	H. Lee Moffitt Cancer Center and Research Institute
18	NCT00960752	II	Active, not recruiting	Melanoma	MAGE-A3	–	Vaccine	M.D. Anderson Cancer Center
19	NCT01266603	II	Active, not recruiting	Melanoma	MAGE-A3	–	Vaccine	M.D. Anderson Cancer Center
20	NCT02787915	I/II	Not yet recruiting	Renal Cell Carcinoma	MAGE-A3, MAGE-A4	–	Antigen Pulsed Dendritic Cells vaccine	Xuzhou Medical University

Hyb3.

Another example of a MAGE-A/HLA specific antibody is 7D4, which was developed by Bernardeau et al. [31]. This mouse antibody was raised not for a therapeutic application, but to facilitate understanding the relationship between density of MAGE/HLA molecules present on the cell surface and efficiency of T cell response.

MAGE directed adoptive T cell therapy

Adoptive T cell therapy (ATC) involves the isolation of peripheral tumor-specific T cells from a cancer patient, their *ex vivo* expansion and re-infusion into the patient with the aim to directly kill cancer cells. Adoptive immunotherapy in which non-gene-modified T cells are being used for cancer treatment has been shown to induce complete and durable responses in patients with metastatic melanoma [32]. The treatment usually involves therapeutic infusion of *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) with high IL-2 regimen after non-myeloablative lymphodepletion. The downside of this approach is that generation of TILs is not always possible, because either they are not present in sufficient quantities, or the *ex vivo* expansion is hampered. Furthermore, there has been limited success in generating TILs from other cancer types than melanoma. The TIL therapy, similarly to the donor lymphocyte infusion therapy for treatment of relapsed leukaemia, represents a more general immune stimulatory type of treatment and does not direct the immune response towards pre-defined tumor antigens [33]. T cells can, however, be re-directed towards tumor antigens when genetically modified to express a T Cell Receptor (TCR) or a Chimeric Antigen Receptor (CAR) of desired specificity. In the following sections we will discuss (pre-) clinical study outcomes of these two types of MAGE-directed adoptive immunotherapies.

MAGE-A directed adoptive T cell therapy using TCR engineered T cells

Already more than two decades ago it has been shown that T cells can be genetically re-directed to recognize and lyse cancer cells [34]. These findings have led to development of more efficient transduction systems generating genetically modified T cells with more stable and durable transgene expression levels [35,36]. Retroviral or lentiviral vectors encoding tumor antigen specific TCRs are now being used to genetically modify T cells with high specificity for many different tumor targets [37]. Despite the fact that adoptive T cell therapy using TCR engineered T cells against tumor-associated antigens has been shown to give successful and durable clinical responses, T-cell-mediated toxicities have occurred because of (low) target expression on healthy tissue [38,39]. This is referred to as 'on-target, off-tumor toxicity'. This type of toxicity could be prevented by choosing a target antigen of which the expression is restricted to tumor tissue [40]. It was postulated that to prevent these side effects, MAGE antigens which are not expressed in normal tissue, except for testis and placental cells, may be the target of choice for this type of therapy [41].

A clinical trial in which nine cancer patients (seven diagnosed with melanoma, one with synovial sarcoma and one with oesophageal carcinoma) were treated with autologous TCR engineered T cells targeting the HLA-A2 restricted CT antigen MAGE-A3 (amino acid 112–120; KVAELVHFL) showed encouraging results. A measurable clinical response was observed in five patients. Two of which were ongoing after more than 12 months. However, three patients developed severe neurological toxicity and two of these patients eventually died because of the treatment. Authors explain this toxicity by possible TCR cross-reactivity with a highly homologous MAGE-A12 derived epitope (KMAELVHFL), which was found to be unexpectedly expressed in a subset of cells in the human brain (NCT01273181) [42].

In another trial in which patients were treated with T cells expressing an affinity enhanced HLA-A1 restricted MAGE-A3 specific TCR an unforeseen severe cardiac toxicity was observed. This toxicity was due to recognition of an epitope (ESDPIVAQY) derived from an unrelated striated muscle-specific protein called titin, which is expressed in the myocardium (NCT01352286) [43,44].

In a phase I dose-escalating study, T cells expressing a MAGE-$A4_{143–151}$ (NYKRCFPVI) specific TCR have been safely applied to patients with recurrent oesophageal cancer. In 5 of the 10 patients the T cells persisted for more than 5 months and retained *ex vivo* antigen specific tumor reactivity. No tumor regression was observed, but three patients exhibited stable disease for more than 20 months. Patients enrolled in this study did not receive lymphodepletion prior to the treatment. In adoptive T cell therapy, lymphodepletion is applied in order to reduce the number of immunosuppressive cells and to reduce competition for activating cytokines (UMIN000002395) [45].

Multiple clinical trials using TCR engineered T cells targeting MAGE-A3, -A4, or -A10, are currently ongoing (Table 2). Altogether, MAGE-directed T cell trials have shown clinical efficacy in a subset of patients and provided more insight with respect to study design, choice of target and safety. Together with the outcome of ongoing trials, this will undoubtedly further increase clinical efficacy and safety for MAGE directed adoptive T cell therapy in the near future.

Several preclinical studies involving MAGE derived epitopes have been published. One study reported the identification of two MAGE specific TCRs from melanoma patients who responded to MAGE vaccination. One TCR is HLA-A2 restricted and recognizes the MAGE-$C2_{336–344}$ peptide (ALKVDVEERV), the other TCR is HLA-DP4 restricted and recognizes the MAGE-$A3_{243–258}$ peptide (KKLLTQHFVQENYLEY). The authors intend to start testing these TCRs in a phase I clinical trial [46]. More recently, another group reported the isolation of a DP4 restricted TCR recognizing the same MAGE-$A3_{243–258}$ peptide [47]. This TCR was tested in a phase I dose escalation study in patients with different metastatic cancers. Among seventeen patients who were treated, one complete response was observed in a patient with cervical cancer (ongoing ≥ 29 months) and three patients receiving the highest dose level showed partial response (ongoing ≥ 19 months). This study shows clinical efficacy and safety of genetically engineered MHC class II restricted MAGE-A3 specific T cells (NCT02111850) [48].

Another preclinical study demonstrated that TCR gene therapy with an HLA-A24 restricted TCR against previously described MAGE-$A4_{143–151}$ peptide (NYKRCFPVI) is a promising strategy to treat patients with MAGE-A4-expressing tumors. They showed in this study that genetically engineered T cells expressing this MAGE-A4 specific TCR could inhibit the growth of MAGE-A4-expressing oesophageal tumors in immunodeficient NOG mice [49].

MAGE directed adoptive T cell therapy using CAR engineered T-cells

Isolation of tumor antigen specific TCRs for adoptive T cell therapy is laborious and may not always be possible. To circumvent the need of tumor specific TCRs, T cells can be genetically engineered to express a chimeric antigen receptor (CAR). CARs are antibody-based recombinant receptors which are anchored in the T cell membrane by fusion to a transmembrane domain and a cytoplasmic signalling domain which allows T cell activation [50,51]. The CAR concept was first described by the group of Zelig Eshhar already in 1989 [52]. Normally, T cells recognize a peptide presented via an MHC molecule through their TCR. CAR expressing T cells are able to recognize and kill tumor cells independently of MHC. This can be an advantage in disease situations in which MHC is down-regulated, or in case of defective proteasomal antigen processing [53]. The principle of CAR gene therapy follows the same steps as TCR gene therapy, which include patient T-cell isolation, retroviral or lentiviral CAR transduction, T-cell expansion, and conditioning chemotherapy prior to T-cell infusion.

The scFv antigen-targeting motif from a CAR is usually derived from a mouse mAb. The scFv is most often anchored to the membrane via an IgG1 derived hinge region, which is fused to a transmembrane domain, derived from either CD3ζ, CD4, CD8, or CD28. CARs have been developed in three generations, and differ mainly in the composition of the intracellular signalling domain. The first generation CARs are characterized by only one CD3ζ signalling domain, but these molecules

showed transient expression and limited clinical activity [38,54–56]. In order to improve the signalling, an additional costimulatory signalling domain derived from either CD28, inducible costimulator (ICOS), OX-40 (CD134), or 4-1BB (CD137) was incorporated [57]. These second generation CARs provided prolonged *in vivo* T cell persistence and improved anti-tumor activity [58–60]. The third generation CARs include two costimulatory domains (e.g. CD28, OX-40 or 4-1BB) in addition to CD3ζ. Preclinical studies with third generation CAR T cells show increased antitumor efficacy over second generation CAR T cells [61,62]. However, this third generation CARs has an increased risk of "on-target, off-tumor" toxicity by providing for more potent activation signals to the T cell thereby reducing the activation threshold. Several strategies are currently investigated to overcome these toxicities including transient CAR expression [63], inclusion of a suicide gene [64,65] or addition of a targeting molecule designed to remove CAR T cells from the system [66]. So far, most of the clinical trials involving CAR T cells have been performed in patients with CD19 positive haematological diseases. These studies showed promising results, including complete remissions in a majority of treated patients [67–69].

Generation of antibodies with an MHC-restricted specificity paved the way for development of CAR T cells recognizing tumor associated peptides presented by MHC. A preclinical study conducted by Willemsen et al. showed that T cells could be generated, expressing a CAR which specifically recognizes a MAGE-A1$_{160–169}$ derived peptide (EADPTGHSY) in the context of HLA-A1. These CAR T cells were able to specifically respond to and kill MAGE-A1 positive, HLA-A1 positive melanoma cells [70]. In a later study from the same group an affinity matured Hyb3 CAR, showed improved tumor cell killing [71].

Cancer therapeutic vaccines

Another way to employ the patient's immune system to battle cancer is to make use of cancer therapeutic vaccines. Cancer therapeutic vaccines are a type of active immunotherapy designed to delay or reduce tumor growth. Many types of cancer therapeutic vaccines have been employed over the years, including protein or peptide vaccines [72], cell based vaccines, DNA or RNA vaccines [73,74], and vector based vaccines. Cell based vaccines can be autologous tumor cells, but also *ex vivo* generated dendritic cells (DCs), which are exposed to tumor antigen. These cells are used to generate a tumor specific immune response once re-injected into the patient. Vector-based vaccines make use of (live attenuated) viruses [75] or even bacteria [76,77] to deliver tumor antigen encoding DNA into host immune cells in order to evoke an immune response. Most clinical trials evaluating MAGE vaccination were employing protein or peptide based vaccines in which MAGE-A protein or MAGE-A derived peptides were used as an immunogen.

In a phase II clinical trial 36 patients with stage III or IV M1a melanoma were treated with recombinant MAGE-A3 protein combined with two different immunostimulants. Four patients treated with MAGE-A3 combined with the AS15 immunostimulant exhibited objective responses, of which 3 complete responses. Antibodies against MAGE-A3 were found in all patients, but also cellular responses were observed (NCT00086866) [78]. Based on this promising data, other larger MAGE-A3 vaccine based clinical trials were initiated, one of which was a phase III trial in patients with melanoma, called DERMA (NCT00796445). Unfortunately, the objective response rate was lower than in previous studies and the trial was discontinued in 2015. In a more recent phase I/II clinical trial, patients with melanoma were treated with MAGE-A3 Antigen Specific Cancer Immunotherapeutic (ASCI) combined with administration of dacarbazine. While only minor clinical benefit was observed, the treatment was well tolerated and induced a MAGE-A3 specific humoral response (NCT00849875) [79]. Two MAGE-A3 ASCI phase I/II clinical trials in patients with non-small cell lung cancer (NSCLC) proved that it was well tolerated and in both studies an immune response against MAGE-A3 was observed (NCT00290355, NCT00455572) [80,81]. One of these studies was followed up in a the largest phase III trial in lung cancer so far, called MAGRIT (MAGE-A3 as Adjuvant in Non-Small Cell Lung Cancer Immunotherapy). Almost 14,000 patients with resected NSCLC were screened, which resulted in 2312 enrolled and randomly assigned patients. The study was recently discontinued due to failure in meeting its primary objective; as it showed no significant difference noted in disease free survival between MAGE-A3 and placebo groups in patients with MAGE-A3-positive stage IB, II, and IIIA NSCLC (NCT00480025) [82,83].

A phase I clinical trial targeting MAGE-A4 in a patient with metastatic colon cancer reported a significant decrease in tumor growth. The vaccine consisted of an artificially designed long MAGE-A4 derived hybrid peptide consisting of a MAGE-A4 helper epitope (amino acid: 278–299) fused to a MAGE-A4 killer epitope (amino acid 143–154) using a glycine linker. It was demonstrated that the helper epitope mainly stimulated CD4 T cells, whereas the killer epitope induced production of MAGE-A4 specific antibodies. Even though this study was conducted in only one cancer patient, it indicates that long MAGE based peptide vaccine may be beneficial for inducing both cellular and humoral immune responses (UMIN000003489) [84]. In a phase II clinical trial, the safety and efficacy of *ex vivo* expanded T cells primed with a large MAGE-A3 peptide was evaluated in patients with multiple myeloma. The treatment was well tolerated, and clinical responses were observed in the majority of patients, which correlated with the presence of MAGE-A3 specific T cells (NCT01245673) [85].

Two studies using DC's electroporated with MAGE-A3 encoding mRNA reported MAGE-A3 specific cellular immune responses in patients with advanced melanoma [86,87]. In another phase I/II clinical trial patients with relapsed neuroblastoma were treated with a combination of decitabine (5-aza-2′-deoxycytidine, DAC) and autologous MAGE-A1/A3 and NY-ESO-1 peptide pulsed DCs. The treatment was well tolerated and an anti-tumor response was reported in six out of nine patients, with a complete response in one patient (NCT01241162) [88].

One clinical study reported the use of a viral vector based vaccine encoding for MAGE-A1 and -A3 in patients with advanced malignancies. From the 30 treated patients with metastatic melanoma, only 1 patient had a partial response and 2 patients had a stable disease for more than six months [89].

Encouraging results were reported in a phase I dose escalating study in which patients with advanced melanoma are treated with an RNA-lipoplex vaccine encoding for several tumor antigens, among which MAGE-A3. The vaccine is well tolerated and patients developed vaccine specific immune responses. The study is currently ongoing (NCT02410733) [90].

Despite the observed lack of clinical efficacy in the DERMA and MAGRIT study, other vaccination studies, do show anti-tumor responses. Additionally, multiple clinical vaccination studies targeting MAGE-A are currently ongoing (Table 2). Irrespective of the outcome of these studies, they will provide more insight in the choice of antigen, patient stratification, and vaccine design. This will lead to more efficacious and more patient tailored MAGE-A directed vaccination studies in the near future.

Conclusions and future perspectives

While clinical trials directed against MAGE-A antigens so far only show clinical responses in a subset of patients, preclinical data demonstrates great promise for the development of effective treatments against MAGE expressing tumors. Together with the progress made in understanding underlying mechanisms of tumor immune evasion, and the lessons learned from clinical studies regarding safety, this creates a time in which major improvements in the field of cancer immunotherapy can be achieved, with respect to both safety and efficacy.

Regarding safety, in particular two clinical studies directed against

MAGE-A antigens in which unexpected toxicities were observed should be highlighted. Considering that MAGE-A antigen expression is mainly limited to tumor cells, 'on-target, off-tumor' toxicity as reported in the study of Morgan et al. was unforeseen and may be of concern. It was speculated that the expression of MAGE-A12 in human brain led to severe neurological toxicity observed in some patients due to TCR cross-reactivity. MAGE-A12 expression has previously been observed in brain tumors [91], but this is the first study to report low levels of MAGE-A expression in healthy neurological tissues. Further studies are needed to confirm MAGE-A expression in healthy neurological tissues. Of note, in this study a MAGE-A3 directed TCR with enhanced affinity was used, which may have resulted in a reduced T cell activation threshold thereby enhancing the chance of 'on-target, off-tumor' toxicities. In addition to this, it is known that TCRs are promiscuous and that they are able to bind to multiple peptide sequences. One study even showed that a single TCR is able to recognize more than one million peptides [92]. In another clinical study performed by Cameron et al., in which an affinity enhanced TCR was used, off target toxicity towards a homologous peptide expressed in muscle tissue was observed. Therefore, improvement of binding affinity needs to be addressed carefully, as it may compromise specificity, resulting in recognition of homologous peptides presented on healthy tissue.

Therefore, for validation of novel MAGE targets which may be used in immunotherapy studies, extensive preclinical studies are needed to carefully address these specificity issues. Various strategies can be applied to predict binding to peptides with a similar sequence to the target peptide. For instance, amino acid scanning approaches to pinpoint peptide fine-specificity, or crystallography studies can be performed to determine MAGE peptide residues that are involved in TCR, CAR or mAb binding. This allows a more focussed search for homologous peptides. Furthermore, *in silico* studies using MHC prediction programs, and mass spectrometry analysis may indicate whether the peptide of interest can be presented via MHC molecules. In line with this, specificity testing in more biologically relevant culture systems, like primary (tumor) tissue instead of tumor cell lines, should be applied.

To further improve clinical efficacy in MAGE-A directed immunotherapy, different approaches can be taken. In tumor cells it often happens that MHC expression is down-regulated due to promoter hyper-methylation [93]. As MAGEs are intracellular proteins, MAGE derived peptides are being presented to the immune system via MHC molecules. Preclinical and clinical data shows that treatment with a demethylating agent such as decitabine markedly improves not only MHC, but also CT antigen expression in tumor cells [94,95]. Another, more experimental way to circumvent immune evasion through antigen down-regulation, may be by using multiple targeting modalities. For example, bispecific CAR T cells co-expressing HER2 and IL-13Rα2-CARs, demonstrated enhanced *in vitro* and *in vivo* glioblastoma tumor cell killing over T cells expressing only HER2 or IL-13Rα2 CARs [96]. Using bispecific CAR T cells in order to prevent antigen escape may also be applied when targeting MAGE antigens. Another approach to improve clinical outcome is by applying a combination therapy using immune checkpoint inhibitors. Currently, several mAbs targeting immunological checkpoints gained much interest. For instance treatment of certain cancers with Pembrolizumab and Nivolumab, PD-1 pathway blocking antibodies, demonstrated impressive results and gained accelerated FDA approval (see Table 1). A combination therapy of these type of immune checkpoint inhibitors with conventional immunotherapy might be beneficial for treatment of MAGE expressing cancers.

One of the so far unmet needs of the MAGE-A directed immunotherapy relates to the patient stratification. Due to the fact that the target of therapies described in this review is a complex composed of two elements, the expression of both elements should be confirmed prior to start of the therapy. Whereas HLA typing is a routine procedure performed to determine tissue compatibility prior to organ transplantation, the confirmation of pre-defined MAGE-A peptide presentation is more challenging. Currently, two methods are most often employed to assess MAGE-A expression, namely reverse transcription polymerase chain reaction (RT-PCR) and/or immunohistochemistry of resected tissue. The limitation of these methods is that they merely confirm MAGE-A expression, but do not ensure that the appropriate MAGE-A derived peptide is presented via MHC-I. Development of high-throughput, cost-effective, reliable methods to characterize tumor peptidome are eagerly awaited and will contribute to better patient stratification.

More recently, whole exome sequencing allowed identification of tumor specific antigens derived from mutated proteins. These so-called neo-antigens provide patient tailored treatment and may represent more immunodominant targets for tumor immunotherapy. The unique nature of neo-antigens may provide potential in next generation immunotherapeutic therapies, but many obstacles still need to be overcome. For example, improvements in prediction algorithms of neo-antigens are needed and costs involved in patient treatment need to be reduced. An additional challenge concerns clinical approval by regulatory agencies. Each neo-antigen based therapy is unique and carries its own risks and benefits making large randomized trials for these kind of antigens impossible. Because of these challenges, targeting CT antigens will still be justified within the field of cancer immunotherapy.

Taken together, the improved understanding of immunological processes in cancer and the promising clinical data in the field of combinatorial therapies provides great promise for the future of MAGE targeted immunotherapy. The ongoing clinical trials directed against MAGE antigens should provide even more insights into which direction these therapies should evolve.

Conflict of interest

The authors declared that there is no conflict of interest.

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