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REVIEW

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Immunobiology of HER-2/*neu* oncoprotein and its potential application in cancer immunotherapy

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Abstract HER-2/*neu* (also known as HER2 or c-*erb*-B2) is a 185-kDa protein receptor with tyrosine kinase activity and extensive homology to the epidermal growth factor (EGF) receptor. HER-2/*neu* is expressed in many epithelial tumors and known to be overexpressed in approximately 20–25% of all ovarian and breast cancers, 35–45% of all pancreatic adenocarcinomas, and up to 90% of colorectal carcinomas. HER-2/*neu* overexpression represents a marker of poor prognosis. HER-2/*neu*-positive tumor cells are potentially good targets for tumor-reactive cytotoxic T lymphocytes which have been utilized in immunotherapeutic trials. In addition, the "humanized" monoclonal antibody Herceptin has been tested in several clinical trials and proved to be an effective adjuvant therapy for HER-2/*neu*-positive breast and ovarian cancers. Vaccinations aiming at generating T-cell responses are being examined in both experimental and clinical trials. Natural immunity at the level of T and B cells has been observed in patients with HER-2/*neu*-positive tumors confirming the immunogenicity of HER-2/*neu* and encouraging vaccination trials with HER-2 protein–derived subunits or synthetic peptides. This review summarizes recent data from patients with various types of HER-2/*neu*–overexpressing cancers carrying different HLA alleles and exhibiting preexistent immunity to HER-2/*neu*–derived synthetic peptides. It also discusses potential advantages of the various vaccination approaches to immunotherapy targeting the HER-2/*neu* molecule.

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Introduction

Tumor cells may express unique protein structures or molecules shared with normal cells that can be recognized by the immune system. Tumor-specific immunity has been demonstrated both in murine tumor models and by clinical responses in cancer patients after vaccination or following passive immunotherapy with tumor-infiltrating lymphocytes. Recent progress in our understanding of the generation of peptides derived from intracellular proteins and their presentation at the cell surface in the context of MHC class I and class II alleles has led to the identification of several tumor antigens recognized by tumor-specific T cells. The identification of both MHC class I and class II–restricted tumor antigens provides new opportunities for the development of therapeutic strategies against cancer. These tumor antigens have been classified into several categories including differentiation antigens, tissue-specific antigens, mutated antigens, and overexpressed antigens [89]. HER-2/*neu*, a member of this last category, is a transmembrane glycoprotein consisting of a large extracellular domain, a short hydrophobic transmembrane domain, and a cytoplasmic intracellular domain containing both a kinase domain and a carboxyl terminal domain that is autophosphorylated upon receptor activation [94]. The HER-2/*neu* gene, present as a single copy in normal epithelial cells, is amplified by gene amplification in numerous malignant cell types, and its overexpression may contribute to disease initiation and progression [94].

The identification of human epithelial cancer antigens [89] has facilitated the in vitro generation of HER-2/*neu*–reactive cytotoxic T lymphocytes (CTLs). Although the in vitro induction of tumor-reactive CTLs is a procedure that can be performed in a clinical setting there are still some concerns about its application in terms of

effective cancer immunotherapy. For example, HER-2/*neu*–specific CTLs can be detected in breast cancer patients but in most cases do not prevent disease progression [16, 24, 49, 97]. A possible explanation for this observation may be that HER-2/*neu* as a self-antigen induces active tolerance mediated by the deletion of clones recognizing immunodominantly presented antigenic epitopes. However, protein molecules mostly contain subdominant peptides not capable of inducing tolerance and of therefore being immunogenic [73]. Overexpression of HER-2/*neu* may result in high levels of subdominant peptides presented by MHC molecules thereby initiating an immune response [8]. Indeed, reports from experimental models and clinical trials confirm that HER-2/*neu* can be immunogenic and generate antibody production and activation of peptide-specific CTLs and T helper (TH) cells [40]. HER-2/*neu* could thus be considered as a candidate molecule for vaccination studies in patients with HER-2/*neu*–overexpressing tumors although in this case there might be a concern for induction of adverse autoimmune reactions. Clinical data, however, argue against this possibility [18, 19].

Numerous anti-*erb*-B2 monoclonal antibodies have been isolated and some of them are able to inhibit growth of HER-2/*neu*-positive ($^+$) tumors [95]. One such antibody, Herceptin, is now being used in the clinic against metastatic breast cancer with HER-2/*neu* overexpression with favorable results [95]. Clinical results with Herceptin have stimulated interest in developing vaccination strategies to elicit T cell– and B cell–mediated HER-2/*neu*–specific responses. Patients with HER-2/*neu*$^+$ tumors displaying natural T-cell immunity to HER-2/*neu*–derived peptides and also having high HER-2/*neu*–specific IgG titers in their sera may be the best candidates for such immunization protocols. It is anticipated that a better understanding of the actitivies of HER-2/*neu*–specific T lymphocytes (including both CTLs and THs) as well as of anti-HER-2/*neu* monoclonal antibodies will aid passive immunotherapies improving the outcome of clinical trials [27].

The biology of HER receptors

The HER family consists of four genes encoding four homologous HER receptors [65]. These receptors are located on the cell membrane in a variety of tissues. The receptors interact with various growth-factor ligands, which have a common EGF-like motif of approximately 50 amino acids [1]. The HER receptors (designated HER1, HER2, HER3, and HER4) show a similar structure, consisiting of a cysteine-rich extracellular ligand-binding domain, a lipophilic transmembrane part, and an intracellular signal-transducing tyrosine kinase domain which contains a regulatory carboxyl-terminal segment [94]. In contrast to the other HER receptors, HER3 lacks certain residues in the catalytic domain and therefore has a weak kinase activity [34]. HER receptors exist as monomers and their activation usually depends on the presence of their ligands [2]. Upon ligand binding, the four different receptors associate with each other to form ten different dimers, which may be homodimers or heterodimers [3]. Dimers are usually more stable than monomeric receptors. HER1 binds to several ligands including EGF, transforming growth factor α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin [94]. In contrast to HER1, no ligand has as yet been identified for HER2. HER3 and HER4 bind to neuregulins which comprise a family of structurally diverse peptides [3].

HER receptor ligands possess a high-affinity site that binds directly to HER1, HER3, or HER4 and a low-affinity site that recruits HER2 as a heterodimerization partner. Thus HER2 functions as a coreceptor for many ligands and is usually transactivated by EGF-like ligands, resulting in the formation of HER1-HER2 heterodimers whereas neuregulins induce the formation of HER2-HER3 and HER2-HER4 heterodimers [94]. Heterodimers are characterized by a slower rate of ligand dissociation than homodimers and therefore generate more potent transducing signals [3]. Moreover, heterodimers containing HER2 undergo a slower rate of ligand-induced endocytosis compared to other HER receptors and thus have a particularly high signaling potency [35]. The HER2-HER3 heterodimer is the most potent mitogenic combination and is the predominant heterodimer in carcinoma cells.

HER receptor–mediated signaling

In human breast and ovarian cancer cells, overexpression of HER-2/*neu* increases basal receptor tyrosine phosphorylation which correlates with effects on cellular transformation in a dose-dependent manner [12]. Specific tyrosine sites in the carboxyl part of HER-2/*neu* have been identified which may be important for HER-2/*neu* signaling. Importantly, some studies have suggested that deletion of these sites does not entirely compromise the ability of HER-2/*neu* to transform cells or to activate downstream signaling molecules [65]. In general, which sites are autophosphorylated and hence which signaling proteins are engaged is determined by the nature of the ligand and the heterodimeric partner. Phosphorylation events lead to activation of multiple second messengers. Many downstream signaling molecules complex with activated receptor tyrosine kinases (RTK) via src homology 2 (SH2) domains [45]. SH2 domains are present in a number of cellular proteins involved in signal transduction and molecules which function as adaptors for important protein-protein interactions [45]. Many SH2 domain–containing proteins also have src homology 3 (SH3) domains which are also involved in protein-protein interactions [60]. A number of substrates for the HER2 tyrosine kinase containing SH2 and SH3 domains, have been identified in human breast and ovarian cancer. There are three

major intracellular signaling pathways that occur and culminate in transcription of nuclear genes. These include the *ras*/mitogen–activated protein kinase, the phosphatidylinositol-3 kinase route, and the phospholipase C-γ. The immediate early nuclear transcription genes including c-*fos*, c-*jun,* and EGR1 are rapidly upregulated. In breast cancer cell lines, expression of c-*fos*, EGR1, and the early response gene c-*myc* have been found to be induced by anti-HER2 monoclonal antibodies [23, 78]. When HER2 is normally expressed (i.e., not overexpressed), ligands binding to HER receptors form only a few HER2 heterodimers and the HER-2/*neu*–mediated signaling is weak, resulting in normal cell growth. In addition, heterodimeric receptors not including HER2 also provide weak but essential signals for normal cell growth.

Preexistent immunity to HER-2/*neu*

HER-2/*neu* has been considered as a potential target for immunotherapy although it has been assumed that patients would be immunologically tolerant to this nonmutated self-protein. However, Disis et al. [14, 17] have shown that some breast cancer patients with HER-2/*neu* tumors have preexistent T- and B-cell–mediated immunity to the HER-2/*neu* protein. It is important to note that cancer patients exhibiting natural antibody and T-cell immunity to HER-2/*neu* do not develop autoimmune responses, suggesting that HER-2/*neu*–specific antibodies and T cells generated by virtue of HER-2/*neu* overexpression do not recognize basal HER-2/*neu* expression on normal epithelial cells.

Existent antibody immunity to the oncogenic protein has been detailed in patients with breast and ovarian cancer at early stages [17], suggesting that such autoantibodies are induced by the native molecule in a specific manner, based on immune mechanisms similar to those responsible for generating antibodies to foreign proteins, and they do not simply reflect an increased tumor load (which characterizes advanced stages of the disease). Antibody responses have been detected to whole protein and to both the intracellular and extracellular domains. Responses varied between patients, with some patients responding to either the intracellular or the extracellular domains and some responding to both. Usually 10–15% of these patients have high titer ($>$1:1,000) antibodies in their sera whereas the overall percentage of HER-2/*neu* IgG-positive patients with breast and ovarian cancer has been reported to be up to 50–55% [91]. Antibodies to HER-2/*neu* with a titer $>$1:1,000 have been also detected in patients with colon cancer (14%) [91] and prostate cancer (15.5%) [53]. Usually detection of antibodies to HER-2/*neu* correlates with HER-2/*neu* overexpression in patients' primary tumors. Progression of disease apparently suppresses antibody production since only 7% of stage III/IV ovarian and breast cancer patients had detectable HER-2/*neu*–specific IgG [91].

Existent T-cell immunity to HER-2/*neu* has been also detected in patients overexpressing this oncoprotein [14, 18]. This suggests that tolerance to HER-2/*neu* has been circumvented in patients whose tumors overexpress HER-2/*neu*. Natural T-cell–mediated immune responses to self-proteins should be directed against subdominant determinants because dominantly processed self-epitopes should be recognized by high-affinity clones which under normal circumstances are tolerated in the thymus [73]. Overexpression of a self-protein may lead to accumulation of subdominant epitopes on the cell surface of a tumor cell, which can be either directly or indirectly presented (e.g., via dendritic cells [DCs]) to the immune system enabling the generation of cellular immune responses [57]. In vivo peptide vaccinations or in vitro repeated restimulations with peptide-pulsed autologous DCs have also been successfully used for enabling the immune system to develop anti-self responses. There are several reports demonstrating increased frequencies of peripheral blood T lymphocytes from healthy donors and nonimmunized patients naturally responding to melanoma- and prostate cancer–associated peptides [37, 48, 63, 71]. Disis et al. [18] reported that 11% of patients with advanced stage breast and ovarian cancer had preexistent TH cell immunity to HER-2/*neu* measured as specific proliferation in response to stimulation with the proteins' extracellular or intracellular domains (ECD and ICD, respectively). In other reports [19, 41, 42] the vast majority of HLA-A2 patients immunized with peptides derived from potential "helper" epitopes of the HER-2/*neu* protein containing within their sequences HLA-A2-binding "cytotoxic" epitopes, developed both HER-2/*neu* peptide (TH and CTL) and protein (ECD and ICD) specific T-cell immune responses. However, of these patients only a few (up to13%) had preexistent immune responses to some of these HER-2/*neu*–derived peptides which included p369–377 and p689–697.

In those reports, in addition to measuring proliferative responses, preexistent immunity was also detected by sensitizing patients' T cells with peptide-pulsed autologous peripheral blood mononuclear cells (PBMCs) in the presence of IL-2 in short-term cultures followed by enumeration of peptide-specific T cells that secrete IFN-γ in ELISpot assays. By developing a more sensitive in vitro sensitization protocol (i.e., stimulation was performed with patients' peptide-pulsed DCs in the presence of IL-7 and IL-12) we were able to demonstrate a significantly higher percentage (25%) of HLA-A2 patients with preexistent T-cell immunity to p369–377 [80]. Since this peptide also binds to HLA-A3 and HLA-A26, we also examined patients carrying these alleles for p369–377–specific cell precursor frequencies. We found that 30% (3 out of 9) and 60% (6 out of 10) HLA-A26 and HLA-A3 cancer patients, respectively, responded with increased precursor frequencies (range 1:26,500 to 1:72,150) to this particular HER-2/*neu* peptide [80]. Such preexistent, T-cell responses were detected in HER-2/*neu*$^{+}$ patients with various types of cancer, including breast, ovarian, colorectal, lung, and prostate

(Table 1). Furthermore, in patients with the same types of cancer overexpressing HER-2/*neu* we could also detect increased T-cell precursor frequencies to HER-2/*neu*–derived and HLA-A2-restricted cytotoxic peptides p435–443, p665–673, p689–697, p777–785, and p952–960 [81] (Table 2). Patients' PBMCs with increased peptide-specific T-cell precursor frequencies could efficiently lyse autologous DCs pulsed with the same HER-2/*neu* peptide and the autologous HER-2/*neu*–overexpressing tumor cells, suggesting that these peptides are naturally processed and expressed on tumor cells [81].

The detection of preexistent T-cell immunity to HER-2/*neu*–derived peptides measured either as proliferation and IFN-γ secretion or lysis of HER-2/neu^+ autologous tumor cells, suggests that HER-2/*neu* is an immunogenic protein, and active immunization including helper and cytotoxic peptide-epitopes may hold promise. Regimens aiming at costimulation of humoral immunity (i.e., by mixing B-cell epitopes from the ECD with the CTL plus TH epitopes, or by immunizing with longer HER-2/*neu* peptides containing all three epitopes in their sequences) may be more effective. As an alternative, passive immunotherapy by administating anti-HER-2/*neu* monoclonal antibody (mAb) (i.e., Herceptin) combined with active immunotherapeutic regimens could be effective against HER-2/neu^+ cancers.

Table 1 Preexistent immunity to peptide HER-2/*neu* (369–377) in patients with HER-2/neu^+ tumors. Tetanus toxoid–specific CTL precursor frequencies (PFs) in the same patients (range 1:2,500 to 1:10,800). Peptide (369–377)-specific CTL PFs in patients with HER-2/neu^- tumors (< 1:85,000) and in healthy individuals (< 1:102,700)

Type of cancer	HLA-alleles[a]		
	HLA-A2	HLA-A3	HLA-A26
Breast	1:26,000[b] 1:33,000 1:37,100 1:39,800	1:33,500 1:39,800 1:57,900	1:57,900
Colorectal			1:39,900
Lung		1:45,600	1:45,600
Ovarian		1:72,150	
Prostate		1:35,500	

[a]Patients carrying one of the indicated alleles exhibited increased peptide-specific CTL precursor frequencies (PF)

[b]Indicates frequencies of peptide-specific CTL precursors (each sequence corresponds to a single patient tested)

Table 2 Preexistent immunity to HLA-A2-restricted HER-2/*neu*–derived peptides representing CTL epitopes. Tetanus toxoid–specific CTL precursor frequencies (PFs) in the same patients (range 1:2,000–1:9,800). Range of peptide-specific mean CTL PFs of the various peptides in patients with HER-2/neu^- tumors (1:98,500 to 1:176,400) and in healthy individuals (1:153,000 to 1:348,000)

Type of cancer	HER-2/*neu* peptides				
	p435–443	p665–673	p689–697	p777–785	p952–960
Breast	1:13,500	1:12,900 1:13,300	1:16,700	1:11,900 1:17,500	1:4,000 1:14,700 1:18,200
Colorectal			1:14,500 1:19,200		1:13,500 1:15,200
Lung	1:13,900	1:13,300			1:8,200 1:9,800
Ovarian	1:11,100	1:11,900 1:15,600	1:13,500 1:14,900		1:6,000 1:9,900
Prostate	1:17,500	1:14,900			1:13,600

HER-2/*neu*–derived immunogenic peptides

As a general rule, $CD8^+$ CTLs recognize small peptides (8–10 mers) in the context of MHC class I molecules whereas $CD4^+$ TH cells recognize longer peptides (with 10–25 amino acid residues) presented in the cleft of MHC class II molecules [26]. MHC class I and class II proteins function as peptide receptors, with each MHC haplotype optimized to present a large number of structurally different peptides at the cell surface. Some of the peptide residues interact with the MHC class I or class II groove and thus act as anchor residues, thereby defining binding motifs specific for different MHC alleles. Such anchor residues can be used for defining within potential MHC-binding peptides the sequence of a certain protein. For MHC class I and class II molecules the different peptide specificities are described as allele-specific motifs [21, 22, 64]. Thus, numerous different peptides are capable of binding to a given MHC haplotype and represent a natural peptide library.

Proteolytic degradation of intracellular proteins, is mediated by the proteasome complex in the cytosol. The generated peptides are then translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen processing localized on the ER membrane. Loading of peptides onto the MHC class I heavy chain with the assistance of chaperones (calnexin, calreticulin, or tapasin) leads to the correct assembly while the β2-microglobulin and the trimolecular complex via the Golgi apparatus is transported to the cell surface for recognition by $CD8^+$ T cells [84]. Exogenous proteins are processed by APCs through receptor-mediated endocytosis, phagocytosis, or pinocytosis. Proteins are then degraded in endosomes and lysosomes to a heterogenous population of peptides through the action of various proteases responsible for antigen processing. Peptides entering endocytic pathways are loaded onto class II molecules within the specialized lysosomal-like MHC class II compartments, and the MHC-peptide complex migrates to the cell surface for recognition by $CD4^+$ TH cells [28, 84, 87]. Using an approach known as "reverse immunology" it is possible to define, with the assistance of algorithms, protein sequences containing anchor residues for binding to certain MHC class I and class II alleles [44, 68]. Synthetic peptides containing those sequences are then used in vitro for generating peptide-specific CTLs or TH lines and clones. If such peptide-specific cells do not recognize tumor cells expressing the whole protein plus the appropriate allele,

it is apparent that the particular peptide is not naturally processed and presented. Indeed, not all of the putative MHC class I– and class II–binding peptides from a protein are generated in vivo, and especialy in the case of TH peptides it is not easy to predict which peptides will be naturally processed. As an alternative, we and others [6, 7, 31, 47, 52, 56], were able to generate specific T cells by utilizing intact autologous tumor cells (ATCs), total ATC lysates, or eluates from MHC class I or class II molecules expressed on ATCs. Target cells pulsed with tumor protein–derived synthetic peptides were then used to stimulate these ATC-specific T cells and to identify tumor peptide-specific T-cell reactivity. In contrast to the reverse immunology approach, this method has the advantage that the peptides identified are naturally processed and expressed by the ATCs. These techniques have been used to identify several immunogenic peptides of the HER-2/*neu* oncoprotein that are naturally processed and presented. These are listed in Table 3 and several of those are discussed below.

Peptide HER-2 (p369–377) was originally identified by Fisk et al. [24] as an immunodominant HLA-A2-binding epitope recognized by tumor-associated lymphocytes of ovarian cancer. Later on, p369–377 was also found to be expressed by several types of HLA-A2^{+} tumors, including renal cell carcinoma [9], breast carcinoma [42], and melanoma cells [68]. Rongcun et al. [68] was able to generate in vitro T-cell lines and clones from ascitic fluids of HLA-A2^{+} patients with epithelial ovarian cancer recognizing HER-2 peptides (p435–443), (p665–673), (p689–697), and (p952–960) expressed on a variety of tumor cell lines including ovarian, colon and breast carcinomas, and melanomas. The HER-2 (p689–697) was also found to be recognized by gastric cancer–specific CTLs [46]. More recently, we have found that besides classical CTLs, p369–377, p665–673, and p689–697 can elicit NKT cells specifically recognizing their

Table 3 Immunogenic HER-2/*neu* epitopes recognized by CTLs

Peptide	HLA-restricting allele	Reference
HER 5–13	HLA-A2	[36]
HER 8–16	HLA-A24	[36]
HER 48–56	HLA-A2	[38]
HER 63–71	HLA-A24	[36]
HER 106–114	HLA-A2	[46]
HER 369–377	HLA-A2	[24]
HER 435–443	HLA-A2	[36]
HER 654–662	HLA-A2	[97]
HER 665–673	HLA-A2	[68]
HER 689–697	HLA-A2	[68]
HER 754–762	HLA-A3 HLA-A11 HLA-A33	[38]
HER 773–782	HLA-A2	[49]
HER 780–788	HLA-A24	[36]
HER 785–794	HLA-A2	[68]
HER 789–797	HLA-A2	[24]
HER 799–807	HLA-A2	[24]
HER 952–961	HLA-A2	[68]
HER 1023–1032	HLA-A2	[69]

autologous HLA-A2^{+} HER-2/*neu*$^{+}$ ovarian tumors [7]. Peptide HER-2 (p754–762) was shown to induce CTLs from healthy donor–derived PBMCs that were capable of killing the colon tumor cell line SW403 expressing HLA-A3 and HER-2/*neu* [37]. Additional MHC-binding studies with the most common HLA molecules belonging to the HLA-A3 superfamily (HLA-A*1101, HLA-A*3101, HLA-A*3301, and HLA-A*6801) demonstrated that p754–762 was able to bind to four of these five alleles [37]. Eberlein and coworkers [61] identified HER-2 peptide p654–662 from the transmembrane region of this protein as a common epitope presented by various HLA-A27 tumor types, including breast, ovarian, pancreatic, and non–small lung cancer. HER-2 peptides p5–13, p48–56, and p1023–1032 were demonstrated to trigger CTL responses in both HLA-A2^{+} humans and HLA-A2 transgenic mice. Such CTLs lysed HLA-A2^{+} HER-2/*neu*$^{+}$ tumor cells of different origins (breast, colon, lung, and renal cancer) irrespective of the expression levels of HER-2/*neu* [69]. Shiku and collaborators [76] have recently identified two HER-2 peptides, p63–71 and p780–788, capable of inducing HLA-A24-restricted CTL responses against various targets also including HLA-24^{+} HER-2/*neu*$^{+}$ tumor cell lines.

HER-2/*neu* peptides recognized in the context of MHC class II molecules

Despite the emphasis on CTL-mediated immune responses, increasing evidence from both human and animal studies has suggested that optimal cancer vaccines require the participation of both CD4^{+} and CD8^{+} T cells [6]. The essential role of CD4^{+} T cells in antitumor immunity was first shown in animal models, where these cells were clearly demonstrated to provide all necessary stimuli for the induction and maintenance of antitumor CD8^{+} T cells [13, 96]. Reports from cell-based vaccine models against MHC class II-negative tumors [66] indicated that tumor antigens released at the tumor site are taken up by macrophages, processed, and presented to CD4^{+} T cells, which in response, produce and secrete lymphokines that activate tumor-specific CTLs. Moreover, MHC class II knockout mice or mice depleted of CD4^{+} T cells were no longer capable of generating CTL responses against an adenovirus E18 protein epitope, whereas wild-type mice developed helper-dependent CTLs to that particular epitope after cross-priming by antigen-presenting cells (APCs) [72].

The identification of antigens recognized by CD4^{+} T cells on human tumors has placed strong emphasis on the role of CD4^{+} T cells in antitumor immunity. Using peptide-binding prediction algorithms, MHC class I–restricted tumor antigens—including melanoma antigens Melan-A/MART-1, gp100, and tyrosinase; tissue-specific antigen MAGE-3; and cancer-testis antigen NY-ESO-1—were demonstrated to contain MHC class II–restricted epitopes recognized by CD4^{+} T cells [30,

90]. Recently, a genetic approach was developed that enabled the cloning of genes coding for mutated MHC class II–restricted antigens including CDC27, triosephosphate isomerase (TPI), and low-density-lipid receptor fusion protein (reviewed in [89]). TPI was also identified by a biochemical approach [89].

There is now also evidence of the existence of MHC class II–restricted T cell responses to HER-2/*neu*: $CD4^+$ T helper cells from HER-2/*neu*$^+$ breast and ovarian cancer patients can proliferate and produce lymphokines in response to stimulation with HER-2/*neu* recombinant protein or synthetic peptides corresponding to immunodominant regions of HER-2/*neu* such as HER-2 (396–406), HER-2 (776–788), and HER-2 (884–899) [5, 15, 25, 85]. Some of these patients indicated preexistent immunity to these peptides in that they responded moderately after a short-term stimulation period [25, 43]. Most recently, we have shown that HER-2 (883–899) can be recognized by healthy donor $CD4^+$ T cells in the context of four different HLA-DR alleles (i.e., DR1, DR4, DR52, and DR53) indicating a high degree of promiscuity in histocompatibility [62]. Disis and collaborators [18, 19, 41] have identified putative T-helper epitopes of HER-2/*neu* that also contained CTL-specific HLA-A2 binding motifs. Vaccination of breast cancer patients with these peptides increased HER-2/*neu* peptide-specific CTL precursor frequencies. In those studies, responses mediated by HER-2/*neu* peptide-reactive $CD4^+$ T cells were defined on the basis of $CD4^+$ T-cell capacity to respond upon recognition of HER-2 peptide-pulsed APCs or DCs pulsed with HER-2/*neu* recombinant protein, whereas evidence for the capacity of peptide-specific $CD4^+$ T cells to directly recognize HER-2/*neu*$^+$ tumor cells has been lacking. In our recent reports [62, 79] we were able to show that HER-2/*neu* peptides p776–788 and p884–899 specific $CD4^+$ T-cell clones from a healthy donor could recognize tumor cells from patients with metastatic breast, colorectal, and pancreatic cancer in the context of at least three alleles, namely, HLA-DRB5*0101, HLA-DRB1*0701, and HLA-DRB5*0405. The finding that this peptide is presented in the context of three HLA-DR alleles is advantageous since (1) it may induce higher frequency of clones recognizing it and thus a more massive antitumor response; and (2) it offers a broad population coverage. MHC class II–presented epitopes from HER-2/*neu* are listed in Table 4.

Table 4 Immunogenic HER-2/*neu* epitopes recognized by T_H

Peptide	HLA-restricting allele	Reference
HER 62–76	DR4/15, DR51, DR53, DQ6/7	[43]
HER 605–619	DR4/15, DR51, DR53, DQ6/7	[43]
HER 765–783	DR4/15, DR51, DR53, DQ6/7	[43]
HER 776–788	DR51, DR7, DR4	[79]
HER 777–789	DR4	[85]
HER 822–836	DR1/11, DR51, DR52, DQ5/7	[43]
HER 883–899	DR1/11, DR4, DR51, DR52, DR53, DQ6/7	[43]
HER 884–899	DR4	[62]

Current therapies

Effect of anti-HER-2/*neu* mAb 4D5 (Herceptin or TrastuzuMab) in clinical trials

The mAb 4D5 was initially shown to inhibit tumor growth in SCID mice carrying HER-2/*neu*$^+$ tumors and to significantly prolong mouse survival [59, 75]. The humanized form of 4D5, termed Herceptin or TrastuzuMab, contains the complementarity-determining regions of the murine mAb together with the human IgG1 constant regions [11]. Herceptin was demonstrated to have similar in vitro and in vivo effects as its murine counterpart [83]. In xenograft models, Herceptin showed a dose-dependent antitumor activity [77]. The use of Herceptin in clinical trials was recently approved by the FDA. Treatment of advanced stage HER-2/*neu*$^+$ breast cancer patients with Herceptin as monotherapy resulted in a response rate of approximately 20% [70, 74]. Improved therapeutic efficacy was achieved by using Herceptin in combination with chemotherapy [70, 74]. Although the clinical results with Herceptin have been encouraging, a large number of patients failed to respond to treatment and all relapsed.

Immunization of cancer patients with HER-2/*neu* peptide-based vaccines

Vaccines targeting the HER-2/*neu* protein may have wide application and utility in the prevention of disease exacerbation in different types of cancer. Generally speaking, cancer vaccines can be formulated using either intact cancer cells or peptides derived from tumor-associated antigens (TAAs). Cancer vaccines utilizing whole tumor cells have the advantage of providing a patients' immune system with all TAAs presented in the context of various MHC alleles. However, they suffer from the fact that due to the heterogeneity of tumor cells, even among patients with the same type of cancer and due to the imprecise knowledge of each tumor cell characteristics, they are not suitable for evaluating immunological responses. On the other hand, vaccination with synthetic peptides offers the advantage of generating defined immune responses applicable to a broad population of patients carrying the appropriate MHC alleles. However, peptide-based vaccinations can be useless if tumor cells down-regulate their MHC alleles or, even worse, that particular TAA. The design of multiepitope vaccines consisting of peptides from several TAAs presented by various MHC alleles may circumvent this problem.

So far, HER-2/*neu* peptide p369–377 administered in incomplete Freund's adjuvant (IFA) or GM-CSF has been used in most published clinical trials. All the trials have demonstrated no adverse reactions from treatment but have also shown only modest effectiveness. In one

study [98], vaccination with p369–377 in IFA generated in vivo peptide-specific CTLs which were, however, unable to recognize HER-2/*neu*$^+$ tumor cell lines. Murray et al. [55] reported that administration of p369–377 with GM-CSF resulted in positive DTH responses in vivo and significant proliferative responses to this peptide in vitro in most of the patients included in the vaccination study. There were no clinical responses. Using a similar protocol, Knutson et al. [42] showed that vaccination with p369–377 plus GM-CSF results in increased precursor frequencies of peptide-specific CTLs which, however, are of low magnitude and short-lived, not being detectable 5 months after the final vaccination.

DCs pulsed with p369–377 and also p654–662 peptides were used to immunize patients with breast or ovarian cancer [10]. After three vaccinations, peptide-specific CTLs producing IFN-γ and being capable of lysing HER-2/*neu*–expressing tumor cells were generated. Of the six patients immunized one showed stable disease.

Disis and collaborators in two other studies [18, 19] used longer HER-2/*neu* peptides corresponding to putative TH epitopes, also containing encompassed HLA-A2 binding motifs, to immunize patients with breast or ovarian cancer. In most cases, peptide-specific proliferative responses could be detected which were occasionally also directed against the ECD or ICD of the protein. There was also a notable increase in the peptide-specific CTL precursor frequencies. Cytotoxicity or clinical responses to treatment were not reported.

The above-mentioned clinical studies have shown that it is quite possible to induce T-cell responses against HER-2/*neu* peptides in cancer patients, suggesting that peptide immunization may be a means of overcoming tolerance directed at immunodominant epitopes. Despite the induction of peptide-specific T-cell responses in vivo, no clinical responses have been reported. Thus, the fact that a HER-2/*neu* peptide, or in general a TAA-derived peptide, elicits tumor-specific immune responses does not necessarily mean that this response is sufficient to reduce tumor load. Theoretically, to do so, a tumor vaccine should be optimized by including multiple peptide epitopes capable of eliciting strong T-cell responses. A limitation to the use of multiepitope vaccines is the necessity of matching patients' HLA haplotype with allele-specific peptides. This means that broad application will require multiply different vaccines. Peptide-based vaccines generally do not elicit antibody responses, which are important for mounting effective anti-HER-2/*neu* responses. Thus the combination of active immunization with the infusion of anti-HER-2/*neu* antibodies (i.e., Herceptin) may induce better clinical results. Protein- or protein subunit–based vaccines encompassing multiple helper and cytotoxic sequences and also stimulating antibody production, offer a good alternative for providing an effective response against HER-2/*neu*–expressing tumors.

Immunotherapy with cytotoxic lymphocytes engineered to express chimeric receptors recognizing HER-2/*neu*

Chimeric receptors facilitate the generation of antigen-specific effector cells independently of the availability of T cells carrying a suitable natural T-cell receptor (TCR), and allow the bypassing of MHC-restricted recognition of peptide antigens as a requirement for the initiation of cytolytic effector functions. This might help to overcome some of the limitations inherent to adoptive transfer of tumor-infiltrating lymphocytes such as heterogeneity of effector cell populations and poorly defined target specificity. Chimeric antigen receptors are composed of a single-chain antibody fragment (scFv) fused to signaling components (ζ chain) of the TCR-CD3 complex [50, 54, 58] or to the γ chain of the Fc receptors for IgG [20, 33, 67] or IgE [32, 92]. Introduction of the chimeric genes into T cells enable them to respond in an MHC-independent fashion to an antigen-specific trigger via these receptors by cytokine production [50, 54, 58] and tumor cell lysis [20, 33, 92]. Chimeric receptors recognizing HER-2/*neu*$^+$ tumor cells have been reported by Moritz et al. [54] and Altenschmidt et al. [4], who linked the ζ chain of the TCR with a scFv derived from a mAb directed against the human ErbB-2 receptor [93]. The scFv (ErbB-2)/ζ fusion genes were stably expressed in murine T lymphocytes which subsequently could recognize and lyse either mouse cell lines transfected to express the human ErbB-2 receptor [4, 54] or the human breast cancer MDA-MB453 cell line constitutively expressing the same receptor [54]. This chimeric construct was recently also used for redirecting a human NK cell line against HER-2/*neu*$^+$ tumors [86].

Most recently [29, 51], we constructed two novel HER-2/*neu*–recognizing chimeric receptors by fusing a scFv derived from an antihuman HER-2/*neu* mAb produced by the HB8696 hybridoma with the γ chain of the Fc(γ)RIII or ζ chain of the TCR. Such chimeric genes were stably transduced into the murine MD.45 CTL (H-2^b) hybridoma cell line that could specifically recognize and lyse in vitro HER-2/*neu*–expressing human tumor cell lines from four different types of cancer (i.e., breast, ovarian, renal, and colorectal). The same cell lines were highly aggressive in vivo in that they formed solid tumors in short periods of time when inoculated in SCID mice. Injection of transduced MD.45 CTLs into these mice significantly prolonged their survival. In a syngeneic mouse-tumor model, the grafted MD.45 CTL effectors protected C57BL.6 (H-2^b) mice from the growth of syngeneic leukemic ALC tumor cells transfected to express human HER-2/*neu* [51]. In addition, our data [29, 51] and those from Uherek et al. [86] suggest that employing retargeted cytotoxic cell lines for adoptive transfer in clinical trials might help to overcome some of the current limitations (i.e., requirement for efficient transduction of patient-derived effector cells and expansion in quantities sufficient for therapy [82, 88]) and could result in the development of more generally applicable cell therapeu-

tics. These data hold promise for the use of our scFv (anti-HER-2/*neu*)/γ chimeric receptor in gene-therapy approaches to cancer treatment.

Conclusions

HER-2/*neu* is a compelling cancer vaccine candidate because it is overexpressed on cancer cells relative to normal tissues. Several immunogenic peptides from the HER-2/*neu* sequence have been identified and successfully used for generating specific T-cell responses in vitro and in vivo. Future work will show whether such HER-2/*neu*–specific T cells are relevant to tumor eradication in vivo and which will be the optimal vaccination protocol for generating and mobilizing such T cells. Another issue that must be examined is whether active immunization should be applied after standard surgical therapy and chemotherapeutic regimens.

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