HLA-E restricted monoclonal antibodies: Therapeutic potential as a double-edged sword against tumor progression

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Abstract

HLA-E is overexpressed in tumor tissues and shed into the circulation. Two alleles (HLA-E*01:01 and HLA-E*03:01) are common among different ethnic groups. They interact with inhibitory receptors (NKG2 and CD94) present on NK cells and subsets of cytotoxic T cells (CTL), and inhibit their cytotoxic capabilities. The beneficial impact of CTL tumor infiltration is neutralized in the cancer patients with strong HLA-E expression. When HLA-E expression is low the tumor CTL infiltration is associated with better survival. Immunotherapies to mask HLA-E on tumor cell surface with HLA-E restricted (monospecific) mAbs would be beneficial. Commercial anti-HLA-E mAbs (e.g. MEM-E/02 and 3D12) are not specific for HLA-E for they cross-react with HLA-I antigens. There is a need to document monospecificity of anti-HLA-E mAbs by examining their affinity to HLA-Ia alleles and by inhibition of their binding to HLA-E with HLA-E restricted peptide sequence(s) that also contains CD94 and NKG2a binding sites. Possibly, HLA-E restricted mAbs (e.g. TFL-033/-034/-73/-74/-145) together with a polyreactive mAb (e.g. MEM-E/02, TFL-006/-007)) can be used to distinguish the phenotypic expressions of HLA-E during tumor progression. The incidence and intensity of HLA-E-restricted staining in the early stages, in poorly or non-differentiated and non-nodal lesions and in diffuse gastric carcinoma is much greater than that of polyreactive anti-HLA-E mAbs. MEM-E/02 failed to stain diffuse carcinoma consistently while TFL-033 showed strong reactivity. Interestingly, HLA-E restricted mAbs are also trigger the proliferation of non-activated and activated CTLs. Therefore, the monospecific anti-HLA-E

mAb can serve as a double-edged sword to fight cancer. They have potential to bind to HLA-E restricted epitopes recognized by CD94 and NKG2A and block their interaction with HLA-E to restore the cytotoxic capabilities of NK cells and CTLs. In addition, the HLA-E restricted mAbs can simultaneously induce proliferation of both activated and unactivated CD8+ cytotoxic T cells.

1.0. Introduction

Human Leukocyte antigen-E (HLA-E) is a non-classical HLA belonging to the major histocompatibility complex (MHC) family in humans. The MHC consists of several genes located on chromosome 6 (6p21.31) [1, 2]. The MHC class I and class II genes generate HLA. Both HLA-I and-II proteins occur as trimeric complexes on normal cells. HLA-I consists of an HLA heavy chain polypeptide (HC), β2m and a peptide. Activated immune cells (T and B lymphocytes in particular) and human cancer cells may express HLA molecules without β2m [3-7]. HLA-II consists of two heavy chain polypeptides and a peptide. HLA class I is divided into highly polymorphic classical HLA-Ia (HLA-A, with 3913 alleles & 2747 proteins, HLA-B with 4765 alleles & 3465 proteins, and HLA-Cw with 3510 alleles & 2450 proteins) loci in contrast to less polymorphic non-classical HLA-Ib loci [8]. In contrast to HLA-Ia, HLA-Ib molecules, which include HLA-E, have a strikingly restricted tissue distribution [9-11].

Though HLA-Ib trimers bind to peptides for antigen presentation similar to HLA-Ia, they are proven to be associated with immunomodulatory functions associated with natural killer (NK) cells, macrophages, dendritic cells, naïve/effector T and B lymphocytes and antibodies [12-13]. They elicit activation or inhibition signaling cascades by interacting with receptors including CD94/NKG2, Ig-like transcript 2 (ILT2), Ig-like transcript 4 (ILT4), KIR2DL4, and CD160 [14]. Among the different HLA-Ib molecules, HLA-G has been more extensively investigated and is found to be represented by 54 alleles and 18 proteins [15]. HLA-F is less studied, and it is currently found to be represented by 22 alleles and 4 proteins. HLA-E is represented by 25 alleles and 8 proteins. In order to have a better understanding of the individuality of HLA-E, its role in tumor progression and the role of anti-HLA-E antibodies in immunodiagnosis and immunotherapy, a holistic perspective of all of the HLA-Ib molecules are presented.

1.1 HLA-G

The alternate splicing of the HLA-G primary transcript generates seven different mRNAs and encode the same number of isoforms. They include four membrane-bound (HLAG1, G2, G3 and G4) and three soluble proteins (HLA-G5, G6 and G7) [16]. The alternative splicing is directly related to the production of soluble and truncated proteins, and the expression of a specific HLA-G isoform will depend on the cell type and location [17-19]. The extracellular structure

of HLA-G1 and HLA-G5 is identical to other HLA-I trimers. The other isoforms can be dimers with or without β2m or presenting peptides [20, 21]. HLA-G1 and HLA-G5 are found in healthy tissue, and there is also a shed soluble HLA-G1 isoform. This shedding is dependent on metalloproteinase activity, at the post-translational level, and it is regulated by different levels of nitric oxide concentration and the activation of Tumor Necrosis Factor – alpha/NFkB [22, 23]. The HLA-G is capable of forming dimers, which show higher affinity and slower dissociation rates, compared to the monomers [24, 25]. The dimerization of HLA-G is a result of the presence of two unique cysteine residues, located in position 42 of the alpha-1 domain and in position 147 of the alpha-2 domain [24]. The HLA-G molecules have the same capability to bind inhibitory receptors, just like the HLA class I molecules [26-28]. Three well known HLA-G receptors are immunoglobulin-like transcript 2 (ILT2) (CD8 5j/LILRB1), ILT4 (CD85d/ LILRB2) and the killer cell immunoglobulin-like receptor (KIR) 2DL4 (CD1 58d) [26-28]. Soluble HLA-G was shown to inhibit different immune-cell populations. The interaction of HLA-G with immune effector cells is mediated by ILT2 on T cells, B cells, NK cells and antigen-presenting cells (APC), by ILT4 on APC (myeloid cells), by KIR2DL4 on T cells and NK cells, and by CD160 on T cells, NK cells and endothelial cells [29, 30].

1.2. HLA-F

HLA-F has 22 alleles, encoding 4 proteins that are known to exist [15]. Unlike HLA-Ia, it has an intracytoplasmic domain [31]. Although HLA-F associates with β2m [32], no information is available on peptide presentation. HLA-F also occurs in circulation as sHLA-F [33] as in other sHLA-I molecules. HLA-F expression is upregulated by IFN-γ. HLA-F expression is also inducible by nuclear factor κB (NFκB) through the κB1 site of enhancer A, located in the proximal promoter region [34]. No cell-surface expression of HLA-F is observed on any of the resting T-cell, B-cell, or NK-cell subsets despite the presence of intracellular HLA-F [35]; however, it is upregulated on activated lymphocytes [35]. The intracellular expression of HLA-F is independent of lymphocyte activation, but surface expression is promoted upon activation. Only T memory cells but not regulatory T cells express HLA-F upon activation. The HLA-F molecule is known to bind to the surface of monocytes [36] and to a subpopulation of CD19+ B cells, but not to CD56+ NK cells or CD3+ T cells.

HLA-F was shown to bind to ILT2 and ILT4 receptors [36, 37]; however, the interaction with ILT2 and ILT4 was only partially inhibited by monoclonal antibodies (mAbs) specific for ILT2 and ILT4. So HLA-F may also interact with other receptors expressed on B cells and monocytes [37]. HLA-F may also dimerize or combine with β2m-free HLA-Ia to present exogenous peptide and/or to interact with NK cells [15, 38-40].

1.3. HLA-E

HLA-E is expressed in most tissues at low levels. Although more than 15 alleles are known to exist, only two are extensively distributed in different ethnic groups [41]. Both alleles differ in only one amino acid at the peptide binding region at position 107 [42-44]; Arginine in HLA-E^{R107} (HLA-E*01:01) is replaced by Glycine in HLA-E^{G107} (HLA-E*03:01) [45]. Such exchange between the two amino acids at position 107, renders differential thermal stability when the bind to the same peptide, resulting in a more stable expression of HLA-E*01:03 on the cell surface compared to HLA-E*01:01 [44]. Higher thermal stability also potentially influences the half-life of the molecule. HLA-E*01:01 and HLA-E*03:01 bind to different restricted sets of peptides. The cellular origin of the peptides are diverse. They include peptides derived from both nuclear as well as from cytosolic proteins. The role of HLA-E is to present peptides derived from HLA-Ia signal sequences (leader peptides), heat-shock protein (Hsp-60), human cytomegalovirus, Hepatitis C virus, Human Immunodeficiency virus, Eptein Barr virus, Influenza virus, Salmonella enteric and Mycobacterium glycoproteins to T-lymphocytes [46] A typical example of some of the peptides are shown in **Table 1** [47]. Recently, a more extensive list of the peptide ligands of HLA-E alleles was presented elsewhere [48].

2.0 Factors influencing the expression of HLA-E on Human cells

Most importantly, HLA-E can be overexpressed in the tissues and shed into the tumor microenvironment and circulation as soluble HLA-E (sHLA-E) [49-55]. The sHLA-E is capable of inducing autoantibodies in humans. An early report on the expression of HLA-E in human cancer has focused on the expression of HLA-E gene in two human embryonic and extraembryonic tumor cell lines, the teratocarcinoma Tera-2 stem cells and the human choriocarcinoma cell line Jeg-3, derived from fetal trophoblasts [56, 57]. It was noted that HLA-E is not significantly

transcribed in either cell line; however, the transcription of HLA-E was up-regulated after treatment with IFN-Γ in Tera-2 cells, while the HLA-E gene in Jeg-3 cells remained unresponsive. The HLA-E gene is ubiquitously expressed in another tumor cell line (JAR) of trophoblast origin, while HLA-Ia genes required a demethylation agent for their transcriptional activity [58, 59]. In examining the molecular mechanisms involved in the IFN-Γ-induced transcription of the HLA-E genes, it was noted that IFN-Γ induced transcription of the HLA-E gene is distinct from that of other HLA-I genes [60].

In lymphoblastoid cell line (LCL) 721.221, cellular expression of the HLA-E gene was observed, but surface expression was lacking [61]. However, when a nonamer peptide derived from the signal sequence of HLA-A2 was bound to LCL, 721 221 cells, it promoted HLA-E surface expression without increasing the level of HLA-E heavy chain synthesis. Characterizing the peptide bound to HLA-E, it was noted that the peptide consisting of nine amino acids with methionine at position 2 and leucine in the carboxyl-terminal positions may facilitated the cell surface expression of an HLA-E trimer. These amino acid sequence characteristics of HLA-E binding peptides are shown in **Table 1.** Signal peptides derived from certain HLA-B proteins with threonine in position 2 only marginally up-regulated HLA-E surface expression in 221 cells. An examination of HLA-E peptide binding in the TAP negative cell line 334 indicated that peptide binding to HLA-E was dependent on a functional TAP heterodimer. This study establishes that the formation of a trimeric complex composed of heavy chain, β2m and peptide ligand is a prerequisite for its efficient transport to the cell surface. The afore-mentioned observations indicate that HLA-E binds to the leader peptide derived from the polymorphic HLA-A, HLA-B and HLA-C. The peptide binding stabilizes the HLA-E and enables migration to the cell surface. A functioning TAP is required to transport these peptides into the endoplasmic reticulum, where they can interact with HLA-E. Down-regulation of HLA-A, HLA-B and HLA-C production or inhibition of TAP may prevent stabilization of HLA-E by the leader peptide. When HLA-E does not reach the cell surface and the tumor cell is susceptible to lysis by NK cells. The molecular mechanisms underlying this function of HLA-E is also revealed by crystallographic studies of the structure of HLA-E [62].

3.0 Immunomodulatory role of HLA-E

3.1. Dynamics of HLA-E binding to CD94/NKG2A inhibitory receptors of NK & T cells.

HLA-E interacts with NK cells and subsets of T cells [63-66]. These cells express a potentially functional receptor complex, known as NKG2 and CD94. Depending on the nature of the NKG2 isoform, the NKG2/CD94 receptor complex performs either inhibitory (e.g., NKG2A/ CD94) or lytic (e.g., NKG2C/CD94) roles [67, 68]. HLA-E presents HLA-I leader sequence peptides [69]. The presentation of such peptides to the NKG2A/CD94 heterodimer inhibits NK cell-mediated lysis. For example, the HLA-G leader peptide (VMAPRTLFL) bound to HLA-E is shown to exhibit a several-fold of increase in binding affinity to NKG2A/CD94 compared to NKG2C/CD94 [70]. Analyzing the structure of VMAPRTLFL carrying HLA-E*01:01 bound to NKG2A/CD94, it was noted that the specificity of the interaction is governed by the CD94 subunit [71]. HLA-E ligands are not restricted to the leader peptides of HLA class I molecules [72, 73]. When HLA-E presented a non-leader sequence peptide, ALALVRMLI (from the ATP-binding cassette transporter multidrug resistance-associated protein 7), it potentially inhibited NK cell-mediated lysis. On the other hand, when both HLA-E*01:01 and HLA-E*01:03 were when bound with peptide QMRPVSRVL (which is derived from the leader sequence of human hsp6 and is upregulated during cellular stress), they failed to inhibit NK cell mediated lysis via NKG2A/CD94. This emphasizes the influence of the bound peptide's sequence in the context of this interaction [74, 75]. Human cytomegalovirus (HCMV) is known to downregulate the expression of HLA-Ia expression in order to avoid recognition by the immune system [76]. To overcome NK-mediated lysis of infected cells by NK cells, HCMV upregulates HLA-E. A peptide (VMAPRTLIL) derived from HCMV UL40 [77, 78] that mimics the leader sequence of HLA-Cw03 (VMAPRTLIL) is specifically presented by HLA-E. Presentation of this peptide by HLA-E prevented NK cell mediated lysis. Similarly, a peptide from hepatitis C virus (HCV) stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells [79]. Interestingly, in a HIV positive patient, the presence of a homozygous genotype of HLA-E^{G107} (HLA-E*03:01) was found to be associated with a fourfold decreased risk of pathogenicity of infection compared to a heterozygous or

homozygous HLA-E^{R107} (HLA-E*01:01); this was possibly due to increased NK cell-mediated lysis of infected cells [80]. On the other hand, a peptide (AISPRTLNA) derived from the p24 protein of HIV-1, mimicking an HLA-I leader sequence peptide, is not only capable of binding to HLA-E but also stabilizes its surface expression and facilitates inhibition of NK cell-mediated lysis [81]. These studies suggest two important aspects relevant to inhibition of NK cell-mediate lysis: 1) The peptide-bound HLA-E should be stable on the cell surface to bring about the inhibition and 2) Homozygous or heterozygous peptide bound HLA-E^{R107} (HLA-E*01:01) is more capable of inhibiting NK cell-mediated cytotoxicity than the homozygous peptide-bound HLA-E^{G107} (HLA-E*03:01). Since homo- or heterozygous HLA-E^{R107} (HLA-E*01:01) overexpressed on human cancer cells bind to the inhibitory receptor complex (CD94/NKG2A) to inhibit NK cell-mediated tumor killing, it is hypothesized that they should be targeted by HLA-E specific antibodies to prevent their binding to inhibitory NK cells or cytotoxic T cells.

3.2. HLA-E allows tumor cells to escape from NK & T cell mediated killing.

IFN-Γ treatment of ovarian carcinoma cell lines increased expression of HLA-E mRNA (and HLA-G) at the protein level [82]. Furthermore it resulted in resistance of tumor cells to "NK-like" lysis by peptide- and allospecific subset of CD8(+) Cytotoxic T Lymphocytes (CTLs). Pulsing of untreated OVACs with the leader sequence peptide of HLA-G protected these cells from lysis by CTLs, thus mimicking the inhibitory effect of IFN-Γ. The possible role of HLA-E in providing resistance to tumor cells by NK-cell mediated cytotoxicity was examined in detail in human cancers [83]. HLA-E expression was studied using ant HLA-E "specific" monoclonal antibody 3D12 in a large variety of tumor cell lines that showed well-defined HLA-Ia downregulation. HLA-E was mainly detected in leukemia-derived cell lines. Interestingly, HLA-E expression was related to the availability of free β2m in the cytoplasm of tumor cells. In the melanoma cell lines FM55 and NW145, the downregulation of HLA-Ia paralleled the increase in the cell surface HLA-E. Interestingly, the addition of human β2m to tumor cell lines augmented the cell surface expression of both HLA-E and HLA-G.

Using a common commercial anti-HLA-E mAb (MEM-E/02), HLA-E protein expression was observed on tissue sections of 420 ovarian and cervical cancers, at equal or higher levels than the normal counterpart epithelia in 80% of the tumors [84]. It was noted that the HLA-E expression strongly correlated with components of the antigen presentation pathway, e.g., transporter associated with antigen processing (TAP), endoplasmic reticulum aminopeptide (ERAP), β2m and HLA classes I and II. Furthermore it was noted that in the ovarian cancers, the tumor infiltrating CD94/NKG2A expression CD8+ T lymphocytes (CTLs) are much higher. It is suggested that the immunosuppressive cytokine TGF-β, which is regularly detected in ovarian and cervical cancer [85–87], seems to induce this inhibiting receptor on T cells [87]. In situ detection of HLA-E interacting receptors revealed a very low infiltrate of natural killer (NK) cells, but up to 50% of intraepithelial CTLs expressed the inhibiting CD94/NKG2A receptor. In cervical cancer [88], HLA-E expression did not alter the prognostic effect of CTLs, most likely due to very high infiltrating CTL numbers in this virus-induced tumor. A striking finding of this investigation is that the overall survival of ovarian cancer patients strongly influenced by HLA-E, because the beneficial effect of high CTL infiltration was completely neutralized in the subpopulation with strong HLA-E expression. Interestingly, these results indicate that CTL infiltration in ovarian cancer is associated with better survival only when HLA-E expression is low and that intratumoral CTLs are inhibited by CD94/NKG2A receptors on CTLs in the tumor microenvironment. This is a significant finding necessitating the need to develop immunotherapies to mask with mAbs either the HLA-E overexpressed on cancer cells or to downregulate or block the inhibitory receptors overexpressed on CTLs [89].

4.0. Are the currently used commercial anti-HLA-E mAbs specific for HLA-E?

4.1. Survey of Literature.

Many investigators depended on the commercial mAbs MEM-E/02 and 3D12 for identifying and localizing HLA-E on Human cancers based on the contention that these mAbs specifically identify or recognize HLA-E expression on the cell surface. A survey of the literature, as summarized in **Table 2** revealed that 80% of the publications claiming to have documentation of cell surface expression in cell lines or tumor

biopsies of human cancer used mouse mAb MEM-E/02. The rest of the reports used mAb 3D12 and mAbs MEM-E/06 or E/07or E/08. Hypothetically, there is a need to demonstrate that these mAbs bind to amino acid sequence(s) specific for HLA-E, either by inhibition studies using HLA-E specific peptides or by showing that they do not bind to other HLA class I antigens such as that of HLA-A, HLA-B, HLA-Cw, HLA-F and HLA-G. It is primarily the developers of these mAbs who seem to be unaware of the fact that the non-classical HLA-Ib molecule, HLA-E share several peptide sequence similarities with the heavy chains of classical HLA class Ia (-B and -C) molecules. When the HLA-E polypeptide heavy chain is used as an immunogen to generate these antibodies, there is a strong possibility that several of the mAbs can cross react with other HLA-I antigens if they bind to shared (pubic) epitopes or amino acid sequences.

4.2. Affinity of HLA-E monoclonal antibodies (MAbs) to HLA-Ia molecules.

This hypothesis was by examining the affinity of HLA-E monoclonal antibodies (HLA-E-MAbs) to HLA-Ia molecules and by inhibiting the antibody binding to both HLA-E and HLA-Ia with their shared peptide sequence(s) [131, 132]. Single recombinant HLA molecule-coated beads are used for antibody binding. The antibody binding is evaluated by measuring mean fluorescence index [MFI] with Luminex multiplex flow-cytometric technology. The peptide-inhibition experiments are carried out with synthetic shared peptides, most prevalent to HLA-E and HLA-Ia alleles. The number of HLA-Ia alleles recognized by the HLA-E-mAbs varies with the density of the antigen (quantity of antigen-coated beads) and dilution of the mAb. Binding of HLA-E-mAbs to β2m-free HLAIa antigens confirms the location of the epitopes on the heavy chain (HC) of the antigens. Strikingly, the nature of alleles of HLA-Ia recognized by different HLA-E-mAbs is identical. The binding of HLA-E-mAbs to the HLA-Ia is inhibited dosimetrically by the adjacent peptides, ^{115}QFAYDGKDY^{123} and ^{137}DTAAQI1^{42}, but not by ^{126}LNEDLRSWTA^{135}, another closer shared peptide sequence. The inhibitory peptide sequences in HLA-E are at the α2-helix terminal facing β2m. The HLA-Ia alleles recognized by HLA-E-MAb (e.g., MEM-E/02, MEM-E/06, MEM-E/07, MEM-E/08 [131] and 3D12 [132]), as shown in Table 3, are similar to those recognized by the anti-HLA antibodies found in the sera of healthy non-alloimmunized males [133], in the therapeutic preparations of the purified intravenous immunoglobulin(IVIg) (134,

135), melanoma patients [136] and allograft recipients [137]. These findings further postulated that some, if not all, of the natural HLA-Ia antibodies seen in healthy males and HLA-Ia antibodies found in pre- and post-vaccination and pre- and post-transplantation could be anti-HLA-E, possibly HLA-F and HLA-G antibodies cross-reacting with HLA-Ia alleles. In IVIg, this postulation was verified by testing HLA-Ia antibodies in IVIg after adsorption to an HLA-E conjugated affinity column (134).

4.3. Shared (public) and specific (private) epitopes of HLA-E.

Table 3 compares the amino acid sequences of heavy chains of HLA-E, HLA-F and HLA-G from positions 1 to 250 [138]. Position 107 distinguishes two of the HLA-E alleles as 107R (HLA-E^R) and 107 G (HLA-E^G). Note positions 65 – 70 (six amino acids: RSARDT) in alpha-1 helix and positions 143 – 156 (amino acids 14: (SEQKSNDASE [AE]HQ) in alpha-2 helix and position 180-183 (four amino acids: LHLE) in alpha-3 helix are unique to HLA-E and therefore considered as restricted. Most of the amino acids in the alpha-1 and alpha-2 helical sequences of HLA-E are not found in any of HLA-A, HLA-B and HLA-Cw helices, and hence they are truly HLA-E restricted (**Table 4**). The amino acid sequences 117-123 (AYDGKDY) and 127-135 (NEDLRSWTA), are considered as "shared peptide sequences" because exactly the same sequences are seen in the HLA-Ia (HLA-A, HLA-B and HLA-Cw) and in HLA-Ib (HLA-F and HLA-G). Notably, this is hidden from recognition antibodies and other ligands, when expressed on the cell surface in association with β2m. The same can be said for amino acid sequence 136-140 (VDTAAQI) but D is replaced by V in HLA-F and by M in HLA-A alleles. Although shared amino acid sequences can be found in the alpha-3 domain, the exposure to interaction with external ligands is considered minimal. The summary of HLA-E restricted or HLA-E monospecific (commonly referred to as *private epitopes*) and HLA-E shared amino acid sequences or polyreactive (*non-restricted or public epitopes)* are presented in **Table 5.**

Figure 1A illustrates the location of HLA-E restricted amino acid sequences (RSARDT and SEQKSNDASEA) in the alpha-1 and alpha-2 helices on the HLA-E heavy chain associated with β2m but devoid of peptides. Similarly, **Figure 1B** illustrates the location of shared sequences of HLA-E (AYDGKDY and LNEDLRSWTA) in relation to the position of β2m. Note that the shared amino acid sequences are masked by

β2m and cannot be recognized by external ligands including the monoclonal antibodies, which recognize them in an open conformer; which is when the heavy chain is devoid of β2m. **Figure 2** illustrates another important aspect of HLA-E restricted peptides and their position in relation to the peptides located in the groove of alpha-1 and alpha 2 helices. If a monoclonal antibody is capable of recognizing the HLA-E restricted amino acid sequences, it can still bind to them even in the presence of peptides in the groove, provided they do not mask the amino acids determining the binding specificity of the monoclonal antibodies.

If HLA-E is a cancer biomarker, are mAbs MEM-E/02 or 3D12 monospecific for specific documentation of cell surface expression of HLA-E? Several vendors who advertise these and similar mAbs as "HLA-E specific" depend on immunostaining of these the mAbs to bind to HLA-E heavy chains on the Western blots. But most of them fail to examine whether these mAbs can also bind to the heavy chains of antigens HLA-B or HLA-Cw. In this regard, a group of Italian investigators [139] have taken efforts to undertake such a comparison. It is an elegant study in which the authors have tested MEM-E/02 against purified heavy chains (NP40 lysates) from the cell lines that express HLA-A* 11:01, HLA-B*35, HLA-Cw* 04:01 (CJO), Cw* 05:01 (221 C5) and HLA-Cw* 07:01(221 C7). Indeed, MEM-E/02 bound to A*11:01, B*35, Cw*04:01, Cw^05:01 and Cw*07:01 as shown in an earlier report (130). The authors seems to be content that the MEM-E/02 binding to Western Blots of heavy chains of HLA-A* 11:01, HLA-B*35, HLA-Cw* 04:01, Cw* 05:01, and HLA-Cw* 07:02 is not as intense as the mAb binding to HLA-E heavy chains. The polyreactivity of these MEM-series of antibodies and mAb 3D12 to HLA-Ia and HLA-Ib antigens were examined by using the Luminex Single Antigen bead multiarray assay. HLA antigens representing various alleles on the HLA-Ia beadset are listed on the One Lambda website (http://www.onelambda.com) under "Antibody Detection Products/ LABScreenSingle Antigen Products." The single recombinant HLA-Ia in beadset (cat. #LS1A04, lot 007) include 31 HLA-A, 50 HLA-B and 16 HLA-Cw antigens, together with in-built control beads, coated with human IgG (considered positive control) or serum albumin (Human or bovine) (negative control). The Labscreen beadsets carry not only the HLA-I trimers [heavy chain (HC) with β2m and a specific peptide for each alleles], but also dimers such as HC with β2m, HC with peptide and monomer, HC only

[140]. In addition, the beads coated with heavy chains of HLA-F and HLA-G were also used. **Table 6,** with one of the lots of MEM-E02 and with mAb 3D12, shows in some cases that these mAbs do bind only to HLA-Ia antigens, even though the binding is less than the binding to the heavy chains of HLA-E. Indeed, the Luminex single antigen bead assay is more sensitive and reliable to evaluate the specificity of monoclonal antibodies. The affinity of the antibodies to HLA-Ia antigens was carried on the HLA-Ia Labscreen beadset at different dilutions to confirm the affinity of these antibodies to HLA-Ia alleles (**Table 7**). The HLA-Ia binding affinity of mAbs 3D12 and MEM-E/06 is different from the other MEM-series (E/02, E/07, E/08), revealing that none of these so-called HLA-E specific mAbs are specific for HLA-E, although mAbs MEM-E/02, MEM-E/07 and 3D12 did not bind to HLA-F and HLA-G (**Table 8**). Histopathologists using these mAbs on any tissue and particularly human cancer tissues, should exercise considerable caution when concluding that they are witnessing HLA-E expression or overexpression on these tissues. Often vendors of the mAbs show how elegantly these mAbs bind to HLA-E heavy chains on the western blots without testing whether they bind in similar fashion to other HLA-Ia antigens, particularly HLA-B and HLA-Cw antigens. Although the binding of MEM-E/02 to heavy chains of HLA-B and HLA-Cw antigens were shown on the western blots (133), the authors did not include all the heavy binding alleles indicated in **Table 6.** Unfortunately, while examining the immunostaining in tissue sections of biopsies, either frozen or paraffin, these non-specific mAbs may not truly reflect the presence of HLA-E, unless other documentation of HLA-E gene expression or immunochemical reactivity with HLA-E monospecific mAbs is provided.

5.0. In search of HLA-E monospecific mAbs for specific diagnosis of HLA-E.

5.1. Development of monoclonal antibodies against HLA-E^{G107} and HLA-E^{R107}

With the specific objective of discovering whether there are any anti-HLA-E monospecific monoclonal antibodies, several clones secreting anti-HLA-E MAbs were generated after immunizing a mouse model with two different alleles of recombinant HLA-E^{G107} and HLA-E^{R107} (10 mg/mL in MES buffer) heavy chains, devoid of β2m, obtained from the Immune Monitoring Lab, Fred Hutchinson Cancer

Research Center (University of Washington, Seattle, WA). Immunization of HLA-E HC in mice resulted in both monospecific [130] and polyreactive (cross-reactive with other HLA-I molecules) [134, 135] monoclonal antibodies. The monospecificity of the mAbs was determined by examining their dose-dependent binding to microbeads coated with different alleles of HLA class Ia (HLA-A, -B, and -Cw) and Ib (HLA-E, -F, and -G); A Luminex Single Antigen beadset that contained 31 HLA-A, 50 HLA-B and 16 HLA-Cw antigens was used. A note of caution should be exercised based on the recent documentation that the beadsets carry not only the HLA-I trimers (heavy chain (HC) with β2m and a peptide specific) for each allele, but also dimers such as HC with β2m, HC with peptide and monomer, HC only [140]. In addition, the monospecificity was established by using single antigen beads coated with heavy chains of HLA-F and HLA-G (but not with trimeric HLA-Ib molecules). **Table 9** documents the diverse types of monoclonal antibodies observed after immunizing with heavy chains of HLA-E. Group 1 consists of mAbs that bound to HLA-E only. The rest of the groups reacted with other HLA alleles in different combinations as illustrated in **Table 9**. **Table.10** presents the different monospecific monoclonal antibodies observed. The binding of the mAbs are measured as mean fluorescent intensity (MFI). MFI values below 500 are considered negative. Very rarely were they above MFI 400. Several monoclonal antibodies were tested to determine their antibody strength by measuring MFI at different dilutions. The binding specificity of a selected clone (TFL-033) was determined using the HLA-E-specific or unique peptide sequences that are also the target site of inhibitory receptors of CTLs.

5.2. Immunodiagnositic potential of anti-HLA-E Monospecific mAbs.

Phenotypic expression of HLA-E on the surface of tumor lesions can be of different kinds. It could be a trimer, in the sense that HLA-E is expressed as Heavy chain with β2m and a peptide, or it could be without a peptide as just an intact heterodimer [HLA-E heavy chain with β2m, or it could be peptide free and β2m-free monomer. In the absence of β2m, the heavy chain exposes cryptic epitopes that contain shared amino acid sequences as discussed above (e.g., [115] QFAYDGKDY[123], [137] DTAAQI1[42], and [126] LNEDLRSWTA[135]) (see figures in [133]). , Anti-HLA-E monoclonal antibodies (mAbs), MEM-E/02, E/06, E/07, E/08 and even 3D12 bind to the peptide sequences in β2m-free

HLA-E, which is common and shared with HLA-Ia monomers. The monospecific anti-HLA-E mAb developed at Terasaki Foundation Laboratory, namely TFL-033 [130], and similar such mAbs listed in **Table 11** recognizes HLA-E-restricted peptide sequences (RSARDTA and SEQKSNDASEA) on α1 and α2 helices away from the β2m-site. However it is far from clear whether they can distinguish trimer from β2m containing heterodimer. However, **Figure 3** illustrates the strong possibility of one or more of the monospecific mAbs listed in Table 10 being capable of binding the HLA-E restricted epitopes that contain CD94 and NKG2a binding sites. Therefore, it is hypothesized that one or more of the TFL mAbs can surely bind to peptide bearing HLA-E trimers. Tumor progression may involve the shedding of β2m from HLA-E or the overexpression of β2m-free monomers. There is a need to identify and distinguish the different phenotypic expressions of HLA-E, particularly the intact heterodimer from the β2m-free monomer on the surface of tumor lesions.

Because of the unique peptide-binding affinities of the mAbs, it is hypothesized that TFL-033 and MEM-E/02 may distinguish the phenotypic expressions of cell surface HLA-E during stages of tumor progression. Gastric cancer tissue micro array (TMA) was used to test this hypothesis [114]. Three tissue microarrays were carefully selected for immunohistochemistry. The cores of all TMA were 1.5 mm in diameter and 5lm thick. This investigation documents use of TMA (BN01013a) of normal gastric mucosa (n568), TMA (ST8015) of primary gastric cancer that included 30 adenocarcinoma, 40 diffuse and ten normal gastric mucosa with information on pathological diagnosis, TNM grading and cancer grade. The array included well differentiated, moderately differentiated, poorly differentiated and undifferentiated tumor samples, as well as tumors from stages I to IV. The TMA (ST8013) of the metastatic gastric cancers included five peritoneal metastases, three liver metastases, five ovarian metastases and 27 lymph node metastases. The summary of the findings are presented in **Table 12**. TFL-033 stained diffusely the cytoplasm of normal mucosa. The incidence and intensity of TFL-033 staining of the cell surface in early stages, poorly or undifferentiated and non-nodal lesions and in diffuse carcinoma is much greater than that of MEM-E/02. MEM-E/02 failed to stain diffuse carcinoma consistently while TFL-033 showed strong reactivity. The non-immunostaining by MEM-E/02 and positive reactivity of TFL-033 (**Figure 4**)

indicate the presence of intact HLA with β2m or clustering of cell surface over-expressed HLA-E masking the amino acid sequences shared by HLA-E and HLA class Ia alleles. The MAb MEM-E/02 failed to immunostain because the shared epitopes were cryptic. But since the amino acid sequences on the α1 and α2 helices were exposed and available, TFL-033 alone could recognize the epitopes and show positivity. MEM-E/02 stained terminal stages, adenocarcinoma and lymph node metastatic lesions intensely, either owing to increased expression of β2m-free HLA-E with tumor progression or owing to expression of HLA-Ia molecules. The study evaluated the relative diagnostic potential of HLA-E-monospecific TFL-033 and the HLA-Ia-reactive MEM-E-02 for determining the specific distribution and immunodiagnosis of different phenotypic expression HLA-E in tumor lesions, and the structural and functional alterations undergone by HLA-E during tumor progression. The study is the first to document clearly and confidently the immunodiagnosis of HLA-E in gastric cancer. **Figure 5** illustrates immunostaining of human melanoma microarrays with MEM-E/02 and with monospecific monoclonals in culture supernatants (s) including TFL-033s, -034s, -073s, and -145s, all of them with high isotype-specific MFI (>20 K when tested neat). All monospecific MAbs and MEM-E/02 showed identical staining. While background staining was highly prevalent with MEM-E-02, immunostaining with TFL-MAbs was sharp and distinct. Since both MEM-E/02 and the monospecific TFL MAbs stained similar locations, it was inferred that the MAbs were reacting to β2m-free HLA-E [130]. It is strongly suggested that future investigators using anti-HLA-E monospecific mAbs such as those reported here (**Table 10)**, should be better clarified when the tissue sections are simultaneously tested with monospecific mAbs and polyreactive HLA-E mAbs such as the MEM series mAbs and 3D12. These observations support the contention that the monospecific mAbs such as TFL-033 and others have the potential to block HLA-E on tumor cells and, therefore, can be a potential reagent for passive immunotherapy.

6.0. The immunotherapeutic potential of monospecific anti-HLA-E mAbs

6.1. The HLA mAbs augment both unactivated and activated CD4-/CD8+ T lymphoblasts

The monospecific anti-HLA-E mAbs are also capable of triggering the proliferation of both non-activated and activated CD8+ T lymphocytes, which includes both

CD3+/CD8+ cytotoxic T cells and CD3-/CD8+ NK cells or NKT cells. A significant increase in the number of CD4-/CD8+ T lymphoblasts among the PHA-treated T lymphoblasts was observed [130] under the influence of the monospecific mAbs TFL-033s (at 1/30 and 1/150, and TFL-034s at 1/50), whereas the number of PHA-untreated T lymphoblasts increased for almost all mAbs (TFL-033s at 1/30 and 1/150, TFL-034s at1/10 and 1/50, TFL-073s at 1/50, TFL-074s at 1/10, and TFL-145s at 1/20). The increase in PHA-untreated T lymphoblasts clarifies the functional potential of HLA-E monospecific mAbs in augmenting CD4-/CD8+ T lymphoblasts. These observations are especially striking because a significant increase in the number of CD8+ T lymphoblasts was observed even in the absence of an activating co-stimulant (PHA). This unique property of augmenting the number of CD8+ T lymphocytes is an added benefit and a further indication for the use of monospecific anti-HLA-E mAbs in cancer treatment. The monospecific anti-HLA-E TFL mAbs is indeed capable of binding to HLA-E molecules expressed on the CD8+ T and NKT cells. HLA-E is a component of unspecified HLA class I antigens earlier identified at low levels on inactivated CD8+ cells and upregulated [3,4] or heavily clustered [141] in activated (by PHA or IFN-Γ) CD8+ cells. Both blastic transformation and proliferation result in transitory cell-surface expression of several molecules, including IL-2R, Fc receptors for IgG (FcγRI/CD64, FcγRII/ CD32 and FcγRIII/CD16, IgE (FcεRII)/CD23), insulin receptors; insulin-like growth factor 1R and IL-2R, alpha-fetoprotein and transferrin receptors, a non-disulphide-linked heterodimer of polypeptide chains 33 kDa and 38 kDa called 'Me14/D12', MICA, HLA class II antigens HLA-DR, -DP and –DQ (see 142 for citations] and most importantly, the over-expression of β2m-free heavy chains of HLA class I [92, 96,107]. It is anticipated that the binding of the monospecific anti-HLA-E mAb on to α1 and β2 helices of the overexpressed and clustered open conformers of HLA-E in activated normal human CD8+ T and NKT cells may induce phosphorylation, promoting proliferation of both non-activated as well as PHA-activated CD8+ T and NKT cells. A model illustrating the hypothesis is presented elsewhere [130]. Since NKT cells are devoid of CD3 molecules, it is envisaged that the TFL-mAb mediated activation of CD8+ NKT cells may be independent of CD3 molecules or may involve different cell surface receptors.

6.2 Monospecific anti-HLA-E mAbs can be considered as a Double-edged sword for immunotherapy of Cancer.

The hypothesis that the monospecific anti-HLA-E mab can serve as a double-edged sword for immunotherapy of cancer is based on two facts.

(1) The mAbs can specifically bind to the amino acids on α1 and α2 helices of HLA-E which specifically bind to CD94 and NKG2A (**Figure 3**), therefore they have the potential of blocking their interaction with the inhibitory receptors of the immune cells that have anti-tumor potential. Such blocking may restore the cytotoxic capabilities of CTLs and CD8+ NK cells, which would otherwise remain inactivated by HLA-E on the tumor cell surface. Moreover, blocking HLA-E could be a better strategy than blocking CD94/NKG2A receptors since they continuously recycle from the cell surface through the endosomal compartments and back again to the cell surface, a process that requires energy and the cytoskeleton [143].

(2) The mAbs can simultaneously induce proliferation of CD8+ cytotoxic T cells both unactivated as well as activated ones. In immunotherapy these T cells can be activated various cytokines, such as IL-2.

A most critical issue is to obtained humanized monospecific anti-HLA-E mAbs for passive immunotherapy of melanoma and gastric cancer. The study can further be extended to other human cancers after confirmative documentation of the overexpression of HLA-E using the monospecific anti-HLA-E mAbs.

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Table 1. Some of the established Peptide ligands of HLA-E

V M A P R T L I L	HLA-Cw signal peptide
V M A P R T L F L	HLA-G signal peptide
V M A P R T L L L	HLA-A∗01 signal peptide
V M A P R T L V L	HLA-A∗02 signal peptide
A L A L V R M L I	ATP binding cassette transporter
Q M R P V S R V L	Heat shock protein 60
A I S P R T L N A	HIV gag protein
S Q Q P Y L Q L Q	Gliadin-wheat protein
S Q A P L P C V L	EBV-BZLF1 protein

Table 2. Cell surface expression of HLA-E on human cancer cells (biopsies or cell lines) monitored with mouse anti-HLA-E monoclonal antibodies (MEM-E/02, MEM-E/06, MEM-E/07. MEM-E/08, 3D12, 3H2679 and TFL-033).

	Type of Cancer	**HLA-E mAb**	**Reference**
1*	Melanoma, Cervical Cancer,	*3D12*	Marín R et al. Immunogenetics. 54(11):767-75. 2003 [83]
2	Melanoma	*MEM-E/02*	Derré L et al. J Immunol. 177:3100-7. 2006. [55]
3	Melanoma and other cancers	*MEM-E/07 MEM-E/08*	Allard M et al. PLoS One 6(6):e21118, 2011 [53]
4	Melanoma	*TFL-033 MEM-E/02*	Ravindranath et al. Monoclon Antib Immunodiagn Immunother. 34(3):135-53, 2015 [130]
5	Lip squamousal cell carcinoma	*MEM-E/02*	Goncalves et al. Human Immunol. 77(9): 785-790, 2016 [90]
6	Laryngeal carcinoma	*MEM-E/02*	Silva TG et al. Histol Histopathol.26:1487-97. 2011 [91]
7	Vulvar intraepithelial carcinoma	*MEM-E/02*	van Esch EM et al. Int J Cancer. 135(4): 830-42, 2014 [92]
8	Penile Cancer	*MEM-E/02*	Djajadiningrat et al. J Urol. 193(4):1245-51. 2015 [93]
9	Glioblastomas	*MEM-E/02*	Mittelbronn, M. et al.,J. Neuroimmunol. 189: 50–58. 2007 [94]
10	Glioblastomas	*MEM-E/02*	Kren L et al.J Neuroimmunol. 220:131-5. 2010 [95]
11	Glioblastomas	*MEM-E/02*	Kren L et al. Neuropathology. 31: 129 -34. 2011 [96]
12*	Glioblastoma stem cells	*3D12*	Wolpert et al. J Neuroimmunol. 250(1-2):27-34. 2012 [97]
13*	Glioblastoma	*3D12*	Wischhusen J et al. J Neuropathol Exp Neurol. 64:523-8. 2005 [98]
14	Neuroblastoma	*3H2679*	Zhen et al. Oncotarget. 7(28): 44340-44349, 2016. [99]
15*	Neuroblastoma	*3D12*	Morandi et al. J Immunol Res. 2016:7465741, 2016. [100]
16	Oral Osteosarcoma	*MEM-E/02*	Costa Arantes et al. Oral Surg Oral Med Oral Pathol Oral Radiol. 123(6):e188-e196. 2017. [101]
17	Intraoral mucoepidermoid carcinoma	*MEM-E/02*	Mosconi C Arch Oral Biol. 83:55-62, 2017 [102]
18	Rectal Cancer	*MEM-E/02*	Reimers et al. BMC Cancer BMC Cancer. 14:486.1- 12, 2014.[103]
19	Colorectal carcinoma	*MEM-E/08*	Levy et al. Int J Oncol. 32(3): 633-41. 2008 [104]
20	Colorectal carcinoma	*MEM-E/08*	Levy et al. Innate Immun. 15(2):91-100. 2009 [105]

Table 2: Cell surface expression of HLA-E on human cancer **…**

21	Colorectal carcinoma	*MEM-E/02*	Benevolo M, et al. J Transl Med. 9:184. 2011 [106]
22	Colorectal carcinoma	*MEM-E/02?*	Bossard C et al. Int J Cancer. 131 (4): 855-863. 2012]107]
23	Colorectal carcinoma	*MEM-E/02?*	Zhen et al., Med Oncol. 30(1):482., 2013 [108]
24	Colorectal carcinoma	*MEM-E/02*	Zeestraten et al. Br J Cancer. 110(2):459-68. 2014 [109]
25	Colorectal carcinoma	*MEM-E/02*	Guo et al. Cell Immunol. 293(1):10-6, 2015 [110]
26	Colorectal carcinoma	*3H2679*	Ozgul Ozdemir et al. Ann Diagn Pathol. 25:60-63, 2016 [111]
27	Colorectal carcinoma	*MEM-E/02*	Huang et al. Oncol Lett. 13(5):3379-3386, 2017 [112]
28	Colon carcinoma and leukemia (K562)	*MEM-E/06*	Stangl S et al. Cell Stress Chaperones. 13(2):221-30. 2008 [113]
29	Colon carcinoma	*MEM-E/02*	Zeestraten EC et al. Br J Cancer. 110(2): 459-68.2014 [109]
30	Gastric Cancer	*TFL-033* *MEM-E/02*	Sasaki et al., Int J Cancer. 134(7): 1558-70. 2014 [114]
31	Hepatocellular carcinoma	*MEM-E/02*	Chen et al Neoplasma. 58(5):371-376, 2011. [115]
32	Non-small cell Lung Carcinoma	*MEM-E/02*	Yazdi et al. Oncotarget. 19;7(3):3477-3488, 2016 [116]
33	Breast cancer	*MEM-E/02*	de Kruijf EM et al.J Immunol. 185:7452, 2010 [117]
34	Breast cancer	*MEM-E/02*	da Silva et al Int J Breast Cancer.; 2013:250435. 2013 [118]
35	Ovarian cancer/ Cervical cancer	*MEM-E/02*	Gooden M et al.PNAS USA 108:10656, 2011 [84]
36	Cervical cancer	*MEM-E/02*	Gonçalves MA et al. Eur J Obstet Gynecol Reprod Biol. 141:70-4. 2008 [119]
37	Cervical cancer	*MEM-E/02*	Spaans VM et al., J Transl Med. 10:184. 2012 [120]
38	Cervical squamous and adenocarcinoma	*MEM-E/02*	Ferns et al. J Immunother Cancer. 4:78, 2016. [121]
39	Serous Ovarian Adenocarcinoma	*MEM-E/02*	Andersson et al. Oncoimmunology, 25;5(1):e1052213, 2015.[122]
40	Serous Ovarian Adenocarcinoma	*MEM-E/02*	Zheng et al. Cancer Sci. 106(5): 522–528, 2015 [123]
41	Renal Cell Carcinoma	*MEM-E/02*	Hanak L et al. Med Sci Monit. 15(12):CR638-43. 2009 [124]
42	Renal Cell Carcinoma	*MEM-E/02*	Kren L et al., *Diagnostic Pathology*, **7**:58, 2012 [125]
43	Thyroid cancer	*MEM-E/02*	Zanetti et al. Int J Immunopathol Pharmacol. 26(4):889-96, 2013 [126]

Table 2: Cell surface expression of HLA-E on human cancer**…**

44	Hodgkin Lymphoma	*MEM-E/02*	Kren L, et al., Pathology, Research and Practice 208: 45–49, 2012 [127]
45*	Chronic Lymphocytic Leukemia	*3D12*	McWilliams et al., Oncoimmunology. 5(10):e1226720, 2016 [89]
46*	Chronic Lymphocytic Leukemia	*3D12*	Wagner et al. Cancer, 23(5):814-823, 2017 [128]
47*	Many Cancers	3D12	Sensi M, et al. Int Immunol. 21(3):257-268. 2009 [129]

Table 3. Comparison of amino acid sequences of heavy chains of HLA-E, HLA-F and HLA-G from positions 1 to 250 [Source: 138]. Position 107 distinguishes two of the HLA-E alleles as 107R (HLA-E^R) and 107 G (HLA-E^G). Note positions 65 – 70 (six amino acids: RSARDT) in alpha-1 helix and positions 143 – 156 (amino acids 14: (SEQKSNDASE [AE]HQ) in alpha-2 helix and position 180-183 (four amino acids: LHLE) in alpha-3 helix are unique to HLA-E and therefore considered as restricted. Most of the amino acids in the alpha-1 and alpha-2 helical sequences of HLA-E are not found in any of HLA-A, HLA-B and HLA-Cw helices, and hence they are truly HLA-E restricted. The amino acid sequences 117-123 (AYDGKDY) and 127-135 (NEDLRSWTA) are considered as "shared peptide sequences" because exactly the same sequences are seen in the HLA-Ia (HLA-A, HLA-B and HLA-Cw) and in HLA-Ib (HLA-F and HLA-G). Notably, this is hidden from recognition antibodies and other ligands, when expressed on the cell surface in association with β2m. The same can be said for amino acid sequence 136-140 (VDTAA) but D is replaced by V in HLA-F and by M in HLA-A alleles. Although shared amino acid sequences can be found in the alpha-3 domain, the exposure to interaction with external ligands is considered minimal. Please see the summary of HLA-E restricted and HLA-E shared amino acid sequences in Table 5.

HLA-Ib	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
HLA-E	G	S	H	S	L	K	Y	F	H	T	S	V	S	R	P	G	R	G	E	P	R	F	I	S	V	G	Y	V	D	D	T	Q	F	V	R	F	D	N	D	A	A	S	P	R	M	V	P	R	A	P
HLA-F	G	S	H	S	L	R	Y	F	S	T	A	V	S	R	P	G	R	G	E	P	R	Y	I	A	V	E	Y	V	D	D	T	Q	F	L	R	F	D	S	D	A	A	I	P	R	M	E	P	R	E	P
HLA-G	G	S	H	S	M	R	Y	F	S	A	A	V	S	R	P	G	R	G	E	P	R	F	I	A	M	G	Y	V	D	D	T	Q	F	V	R	F	D	S	D	S	A	C	P	R	M	E	P	R	A	P

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
HLA-E	W	M	E	Q	E	G	S	E	Y	W	D	R	E	T	R	S	A	R	D	T	A	Q	I	F	R	V	N	L	R	T	L	R	G	Y	Y	N	Q	S	E	A	G	S	H	T	L	Q	W	M	H	G
HLA-F	W	V	E	Q	E	G	P	Q	Y	W	E	W	T	T	G	Y	A	K	A	N	A	Q	T	D	R	V	A	L	R	N	L	L	R	R	Y	N	Q	S	E	A	G	S	H	T	L	Q	G	M	N	G
HLA-G	W	V	E	Q	E	G	P	E	Y	W	E	E	E	T	R	N	T	K	A	H	A	Q	T	D	R	M	N	L	Q	T	L	R	G	Y	Y	N	Q	S	E	A	S	S	H	T	L	Q	W	M	I	G

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
HLA-E	C	E	L	G	P	D	R/G	R	F	L	R	G	Y	E	Q	F	A	Y	D	G	K	D	Y	L	T	L	N	E	D	L	R	S	W	T	A	V	D	T	A	A	Q	I	S	E	Q	K	S	N	D	A
HLA-F	C	D	M	G	P	D	G	R	L	L	R	G	Y	H	Q	H	A	Y	D	G	K	D	Y	I	S	L	N	E	D	L	R	S	W	T	A	A	D	T	V	A	Q	I	T	Q	R	F	Y	E	A	E
HLA-G	C	D	L	G	S	D	G	R	L	L	R	G	Y	E	Q	Y	A	Y	D	G	K	D	Y	L	A	L	N	E	D	L	R	S	W	T	A	A	D	T	A	A	Q	I	S	K	R	K	C	E	A	A

	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
HLA-E	S	E	A	E	H	Q	R	A	Y	L	E	D	T	C	V	E	W	L	H	K	Y	L	E	K	G	K	E	T	L	L	H	L	E	P	P	K	T	H	V	T	H	H	P	I	S	D	H	E	A	T
HLA-F	E	Y	A	E	E	F	R	T	Y	L	E	G	E	C	L	E	L	L	R	R	Y	L	E	N	G	K	E	T	L	Q	R	A	D	P	P	K	A	H	V	A	H	H	P	I	S	D	H	E	A	T
HLA-G	H	V	A	E	Q	R	R	A	Y	L	E	G	T	C	V	E	W	L	H	R	Y	L	E	N	G	K	E	M	L	Q	R	A	D	P	P	K	T	H	V	T	H	H	P	V	F	D	Y	E	A	T

	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
HLA-E	L	R	C	W	A	L	G	F	Y	P	A	E	I	T	L	T	W	Q	Q	D	G	E	G	H	T	Q	D	T	E	L	V	E	T	R	P	A	G	D	G	T	F	Q	K	W	A	A	V	V	V	P
HLA-F	L	R	C	W	A	L	G	F	Y	P	A	E	I	T	L	T	W	Q	R	D	G	E	E	Q	T	Q	D	T	E	L	V	E	T	R	P	A	G	D	G	T	F	Q	K	W	A	A	V	V	V	P
HLA-G	L	R	C	W	A	L	G	F	Y	P	A	E	I	I	L	T	W	Q	R	D	G	E	D	Q	T	Q	D	V	E	L	V	E	T	R	P	A	G	D	G	T	F	Q	K	W	A	A	V	V	V	P

Table 4. A comparative study of the amino acid sequences of all HLA class I alleles (both classical and non-classical) showed the unique presence of HLA-E sequences listed below. Theoretically, a monospecific anti-HLA-E mAb is expected to recognize such HLA-E specific amino acid sequences or epitopes.

	α1						α1							
	65	66	67	68	69	70	102	103	104	105	106	107	108	109
HLA-E*01:01	**R**	**S**	**A**	**R**	**D**	**T**	**E**	**L**	**G**	**P**	**D**	**R**	**R**	**F**
HLA-E*01:03							**E**	**L**	**G**	**P**	**D**	**G**	**R**	**F**
HLA-F*01:01	G	Y	**A**	K	A	N	D	M	**G**	**P**	**D**	**G**	**R**	L
HLA-G*01:01	**R**	N	T	K	A	H	D	**L**	**G**	S	**D**	**G**	**R**	L
HLA-A* alleles	No matching sequence						No matching sequence							
HLA-B* alleles	No matching sequence						No matching sequence							
HLA-Cw* alleles	No matching sequence						No matching sequence							

	α2											α2				
	144	145	146	147	148	149	150	151	152	153	154	170	171	172	173	174
HLA-E*01:01/:03	**S**	**E**	**Q**	**K**	**S**	**N**	**D**	**A**	**S**	**E**	**A**	**K**	**Y**	**L**	**E**	**K**
HLA-F*01:01	T	Q	R	F	Y	E	A	E	E	Y	**A**	R	**Y**	**L**	**E**	N
HLA-G*01:01	**S**	K	R	**K**	C	E	A	**A**	N	V	**A**	R	**Y**	**L**	**E**	N
HLA-A* alleles	No matching sequence											No matching sequence				
HLA-B* alleles	No matching sequence											No matching sequence				
HLA-Cw* alleles	No matching sequence											No matching sequence				

	α3				
	180	181	182	183	184
HLA-E*01:01/:03	**L**	**H**	**L**	**E**	**P**
HLA-F*01:01	Q	R	A	D	**P**
HLA-G*01:01	Q	R	A	D	**P**
HLA-A* alleles	No matching sequence				
HLA-B* alleles	No matching sequence				
HLA-Cw* alleles	No matching sequence				

Table 5. Comparison of the amino acid sequence of HLA-E heavy chain polypeptide with the heavy chain polypeptides of HLA-A, HLA-B, HLA-Cw, HLA-F and HLA-G. The peptides are classified as HLA-E restricted and HLA-non-restricted. The HLA-non-restricted peptide sequences are further characterized as polyreactive and HLA-I antigen restricted [Source: 144].

HLA-E peptide sequences [total number of amino acids]	HLA alleles					Specificity
	Classical class Ia			Non-Classical class Ib		
	A	B	Cw	F	G	
^{47}**PRAPWM**EQE^{55} [9]	1	0	0	0	0	A*3306
^{58}**EYWDRE**TR^{65} [8]	5	0	0	0	0	A restricted
^{65}**RSARDT**A^{71} [7]	**0**	**0**	**0**	**0**	**0**	**E restricted**
^{90}**AGSHTL**QW^{97} [8]	1	10	48	0	0	Polyreactive
^{108}**RFLRGY**E^{114} [7]	24	0	0	0	0	A restricted
^{115}**QFAYDG**KDY^{123} [9]	1	104	75	0	0	Polyreactive
^{117}**AYDGKD**Y^{123} [7]	**491**	**831**	**271**	**21**	**30**	Polyreactive
^{126}**LNEDLR**SWTA^{135} [10]	**239**	**219**	**261**	**21**	**30**	Polyreactive
^{137}**DTAAQI**^{142} [6]	**0**	**824**	**248**	**0**	**30**	Polyreactive
^{137}**DTAAQIS**^{143} [7]	0	52	4	0	30	Polyreactive
^{143}**SEQKSND**ASE^{152} [10]	**0**	**0**	**0**	**0**	**0**	**E restricted**
^{157}**RAYLED**^{162} [6]	0	1	0	0	0	B*8201
^{163}**TCVEWL**^{168} [6]	**282**	**206**	**200**	**0**	**30**	Polyreactive
^{183}**EPPKTHV**T^{190} [8]	0	0	19	0	0	C restricted

Table 6. The commercial anti-HLA-E mAbs react to classical HLA-Ia alleles, hence they cannot be considered to monospecific or specific for HLA-E. The mAbs were tested at a dilution of 1/300. Is it not strange that these mAbs have been used in literature as HLA-E specific? See Table 2.

HLA-Ia	mAb	mAb MEM series			
alleles	3D12	E/02	E/06	E/07	E/08
A*0101	88	349	**11453**	251	137
A*0301	789	238	**9525**	177	135
A*1101	166	**2940**	**7067**	**4992**	**1321**
A*1102	121	**559**	**9404**	**580**	102
A*2301	293	358	**12666**	245	220
A*2402	327	**4096**	**14316**	**10991**	**2739**
A*2403	281	**2505**	**15894**	**9981**	**2094**
A*2501	134	**629**	291	373	275
A*2902	195	**1593**	495	**1770**	**643**
A*3001	184	526	**10072**	405	551
A*3002	58	353	**5296**	224	328
A*3201	208	**603**	288	490	241
A*3301	**573**	**3037**	**1195**	**3469**	**1163**
A*3303	228	**1604**	341	**1228**	511
A*3401	122	**991**	**757**	**754**	237
A*3601	103	**1219**	**9451**	**1493**	490
A*6601	140	**571**	126	230	118
A*6801	81	**664**	226	473	184
A*6901	90	**917**	278	376	229
CW*0102	**966**	**3125**	**12748**	**2998**	**3217**
CW*0202	**720**	**2567**	**17083**	**2003**	**1986**
CW*0302	460	**1713**	**14941**	**1705**	**624**
CW*0303	**571**	**2358**	**14248**	**1549**	**1067**
CW*0304	187	**2585**	**13686**	**1875**	**903**
CW*0403	**3796**	**1765**	**7077**	**2052**	**703**
CW*0501	**931**	**9263**	**15923**	**15435**	**6346**
CW*0602	405	**3076**	**17836**	**1914**	**10260**
CW*0702	**1640**	**6680**	**1911**	**7149**	**13655**
CW*0801	**592**	**2481**	**15461**	**2001**	408
CW*1203	**1020**	**1692**	**15372**	**1035**	**771**
CW*1402	480	**1889**	**12570**	**1839**	**1637**
CW*1502	446	**2688**	**16008**	**918**	**1080**
CW*1601	**530**	**1128**	**15803**	**590**	**735**
CW*1701	**5554**	**1869**	**491**	**1361**	**841**
CW*1802	**1095**	**7779**	**14530**	**11223**	**8373**

HLA-Ia	mAb	mAb MEM series			
alleles	3D12	E/02	E/06	E/07	E/08
B*0702	208	**1910**	**12951**	**1375**	**618**
B*0801	361	**1062**	**15204**	246	**4281**
B*1301	**1820**	**5700**	**15934**	**6182**	**3195**
B*1302	**1171**	**1326**	**16249**	393	430
B*1401	127	**3135**	**14876**	**4103**	**1308**
B*1402	138	**942**	**12447**	390	**634**
B*1501	447	**832**	**14606**	367	174
B*1502	224	**3250**	**15676**	**3860**	**1051**
B*1503	85	**4731**	**15401**	**562**	**571**
B*1510	210	**768**	**15482**	358	412
B*1512	371	**1903**	**15670**	**850**	315
B*1513	**591**	**3400**	**15450**	**4023**	**982**
B*1516	334	248	**13159**	112	252
B*1801	126	**4392**	**16138**	**3923**	**1665**
B*2705	248	**942**	**13985**	418	84
B*2708	**3264**	**1175**	**14289**	**640**	125
B*3501	**566**	**8716**	**15768**	**12917**	**6233**
B*3701	154	**3444**	**14356**	**3109**	**1871**
B*3801	**1672**	**968**	**14774**	**516**	468
B*3901	363	**3010**	**12219**	**3825**	**1289**
B*4001	**800**	**3478**	**15269**	**2662**	433
B*4002	**712**	**2442**	**9971**	**2166**	**631**
B*4006	**3216**	**9898**	**15642**	**14269**	**6208**
B*4101	156	**4987**	**14635**	**5400**	**1331**
B*4201	253	276	**14286**	67	102
B*4402	129	**2621**	**15821**	**1525**	216
B*4403	**1321**	**2654**	**13339**	**1625**	376
B*4501	**604**	**3134**	**14671**	**3234**	748
B*4601	419	**3042**	**16173**	**3389**	1062
B*4701	404	**777**	**8849**	341	81
B*4801	244	**3577**	**10982**	223	514
B*4901	67	**1588**	**15804**	490	98
B*5001	292	**769**	**15575**	156	53
B*5101	**841**	**2485**	**13576**	**2619**	**884**
B*5102	349	**2303**	**13138**	**2352**	**800**
B*5201	**1416**	**928**	**8975**	200	210
B*5301	446	**2754**	**13622**	**2890**	**1384**
B*5401	227	**1910**	**13506**	**1274**	**598**
B*5501	**573**	**1287**	**14420**	**892**	180
B*5601	249	**5352**	**16067**	**5075**	**1881**
B*5701	**588**	**3626**	**11746**	**5398**	**1982**
B*5703	**1143**	**2586**	**14723**	**3272**	**1556**
B*5801	**823**	**1636**	**11721**	**1809**	**1155**
B*5901	91	**2803**	**16222**	**1837**	**915**
B*6701	**1856**	**704**	**12533**	136	307
B*7301	**659**	**5560**	**2363**	**8347**	**3629**
B*7801	180	**4273**	**11454**	**5927**	**1678**
B*8101	**579**	**1097**	**11758**	167	347
B*8201	217	**5295**	**15480**	**6315**	**1922**

Table 7. Titration profile of the binding of mAb MEM series E/02, E/06, E/07 and E/08 to HLA-Ia alleles. HLA-1a non-binding alleles of mAb MEM-E/02 is indicated to show the uniqueness of their binding affinity. Note how MEM-E/06 binding of HLA-Ia differs from other members of the series.

	MEM-E/02			**MEM-E/07**			**MEM-E/08**				**MEM-E/06**
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400		1/100
E*01010101	**16978 ± 2291**	**14257 ± 2366**	**12554 ± 1934**		**9778 ± 1935**			**12608 ± 2527**			**10973 ± 2194**
MAb E/02-High Binding Alleles											
B *40060101	5420	2609	1381	4269	1884	692	1906	747	195	B*1402	1206
CW*050101	4400	1806	900	4425	2003	804	1980	815	213	B*5401	1001
CW*1802	3862	1584	806	2924	1232	451	3053	1506	549	B*4501	969
										B*0801	964
B *350101	3789	1527	758	3718	1710	705	1932	802	294	B*1501	964
CW*070101	3501	1855	1006	1163	432	112	4036	1986	739	B*5101	951
B *1301	2786	1215	572	1103	388	74				B*0702	913
B *4101	2361	966	472	980	315	54				B*2708	884
B *5601	2325	1026	510							B*5102	819
B *8201	2230	892	453	960	372	126				B*5701	762
B*1801	1883	817	387							B*5501	718
B *7301	1937	658	302	1768	726	255	947	353	91	B*6701	715
B*4001	1430	657	301							B*5801	708
B*7801	1519	523	206							**B*7801**	742
B*3701	1325	479	146							**B*3701**	1140
B*1513	1260	442	187							B*3901	697
A*2402				1548	570	143				B*4201	694
										A*2301	672
MAb E/02 Non-Binding Alleles										B*4801	657
A*01010101										B*8101	556
A*02010101										B*4701	522
A*020301										B*4002	509
A*7401											
A*03010101											
A*2301											
A*110201											

Table 8 HLA-F and HLA-G reactivities of mAbs 3D12 and MEM series. Note affinity of MEM-E/06 and MEM-E/08 for HLA-F and HLA-G. Both these mAbs are also used as HLA-E specific mAbs in literature. See Table 2.

Heavy chains of non-classical HLA-Ib antigens coated on to Luminex microbeads															
Dilutions	MEM-E/02			3D12			MEM-E/06			MEM-E/07			MEM-E/08		
	HLA-E	**HLA-F**	**HLA-G**	**HLA-E**	**HLA-F**	**HLA-G**	**HLA-E**	**HLA-F**	**HLA-G**	**HLA-E**	**HLA-F**	**HLA-G**	**HLA-E**	**HLA-F**	**HLA-G**
[1/100]	16978 ± 2291														
[1/200]	14257 ± 2366						10973 ± 2194			9778 ± 1935			12608 ± 2527		
[1/400]	12554 ± 1934														
[1/800]	6396 ± 1273			7144	9	8									
[1/1000]	**8585**	**0**	**7**	**6234**	**7**	**7**	**6793**	**2466**	**2504**	**2814**	**116**	**172**	**5435**	**544**	**930**
[1/1600]	2925			4030	8	6									
[1/3200]	957			2109	3	7									
[1/6400]	224														

Table 9. Different groups of monoclonal antibodies generated at TFL after immunizing heavy chains of HLA-E. Only Group 1 is Monospecific whereas the rest of them have varying affinities for HLA-A, HLA-B, HLA-Cw, HLA-F and HLA-G loci.

Groups of anti-HLA-E mAbs	Non-classical HLA			Classical HLA			
	E	F	G	A	B	Cw	
Group 1	+	-	-	-	-	-	24 TFL monoclonal anti-HLA antibodies
Group 2	+	+	-	-	-	-	NONE
Group 3	+	-	+	-	-	-	NONE
Group 4	+	+	+	-	-	-	NONE
Group 5	+	-	-	+	+	+	mAbs MEM-E/02. MEM-E/07 and 3D12*
Group 6	+	+	-	+	+	+	mAb MEM-E/06
Group 7	+	-	+	+	+	+	NONE
Group 8	+	+	+	+	+	+	mAbs MEM-E/06 & E/08; partial HLA-Ia reactivity* mAbs TFL-006 >TFL-006; complete HLA-Ia reactivity**

* For details see Tables 6 to 8; ** For details see Tables and Figures in References 134 and 135

Table 10. Monospecific Monoclonal antibodies generated against HLA-E heavy chain at Terasaki Foundation Laboratory. Two different alleles of HLA-E were used as immunogens. They are HLA-$E^{R107}$and HLA-$E^{G107.}$. The HLA-Ia, HLA-F and HLA-G reactivities of the mAbs are less than <500 and hence regarded as negative. The HLA-Ia alleles showing low and high MFIs values are indicated, as proof for the monospecificity of the mAbs.

	HLA-E^{R107}																		HLA-E^{G107}		
HLA antigen tested	**E^{R}109**	**E^{R}147**	**E^{R}148**	**E^{R}110**	**E^{R}034**	**E^{R}125**	**E^{R}033**	**E^{R}126**	**E^{R}073**	**E^{R}074**	**E^{R}144**	**E^{R}041**	**E^{R}043**	**E^{R}145**	**E^{R}042**	**E^{R}001**	**E^{R}056**	**E^{R}081**	**E^{G}184**	**E^{G}185**	**E^{G}186**
Isotypes	**IgG2b**	**IgG1**	**IgG1**	**IgG2b**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG1**	**IgG2a**	**IgG2a**	**IgG1**	**IgG1**	**IgG1**	**IgG1**
HLA-E^{R}	**23328**	**22829**	**16644**	**16226**	**13272**	**13204**	**13025**	**12397**	**10088**	**10269**	**9180**	**8914**	**8448**	**7622**	**7452**	**4691**	**4581**	**4261**	**19227**	**19644**	**19075**
HLA-F	2	5	3	2	29	3	37	5	12	11	3	8	10	25	8	9	33	9	21	24	29
HLA-G	1	2	1	1	56	2	52	2	9	10	1	9	8	22	6	6	26	6	25	38	106
A* (31 alleles)																					
A*2902	41	30	29	40	46	31	54	35	31	33	0	32	36	33	32	38	46	31	41	39	41
A*2901	287	267	277	282	359	303	402	310	311	316	0	319	329	266	326	349	309	303	55	59	59
B* (50 alleles)																					
B*1502(B75)	213	204	215	208	247	226	241	227	236	239	0	248	253	206	246	274	251	233	22	21	22
B*5703(B57)	16	12	12	17	15	14	12	15	12	13	0	13	14	15	13	16	24	14			
B*1513(B77)	298	305	321	299	380	338	381	353	360	352	0	372	372		372	396	358	344			
B*4006(B61)											0			346							
B*4701(B47)											0								197	198	204
Cw* (16 alleles)																					
CW*0304											0								42	40	
CW*0401											0								42	40	41
CW*1203	43	34	37	41	43	39	37	41	36	36	0	38	40	40	36	45	51	34			
CW*1601											0								42		
CW*1701	192			195	266		252		248	250	0				252			243	131	131	134
CW*1802		495	359			380		439			0	284	305	490		452	444				50

The monoclonal antibodies were produced following the guidelines in the report generated by the National Research Council's Committee on Methods of Producing Monoclonal Antibodies. The recombinant polypeptides of HLA-ER107 (heavy-chain only; 10 mg/ml in MES buffer) were obtained from the Immune Monitoring Lab, Fred Hutchinson Cancer Research Center (University of Washington, Seattle, WA). Each antigen was immunized in two different mice. Fifty micrograms of the antigen (β2m-free heavy chain) in 100 µl of PBS (pH 7.4) were mixed with 100 µl of TiterMaxVR Gold adjuvant (CytRx, San Diego, CA) before injection into the footpad and intraperitoneum of the mice. Three immunizations were given at about 12-day intervals. The clones were cultured in a medium containing RPMI 1640 w/glutamine and sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, cat. no. R8758), 15% fetal calf serum, 0.29 mg/ml L-glutamine/Pen-Strep (Gemini-Bio, MedSupply Partners,Atlanta, GA, cat. no. 400-110) and 1 mM sodium pyruvate (Sigma, cat. no. S8636). Several clones were also grown using Hybridoma Fusion and Cloning Supplement (HFCS) (Roche Applied Science, Indianapolis, IN, cat. no. 11363735001). Isotypes of the mAbs were characterized, and no IgM Abs were detected.

Table 11. Inhibition of purified culture supernatants of TFL-033 with two HLA-E-restricted peptides, ^{65}RSARDTA^{71} and ^{143}SEQKSNDASE^{152}, at concentrations of 0.27 µg/well. Although both peptides showed inhibition, the α2 helical peptide SEQKSNDASE showed better inhibition than the other peptide.

	mAb TFL-033 (IgG1)	Protein G purified Culture supernatant	
mAb Concentration	0.07 µG/14µG/well	0.07 µG/14µG/well	0.07 µG/14µG/well
Peptide	none	**^{65}RSARDTA^{71}**	**^{143}SEQKSNDASE^{152}**
Peptide concentration	none	0.27 µG/14µG/well	0.27 µG/14µG/well
Mean (n= 3)	3759	3371	
SD ±	118	172	
p^2		0.03	
Mean (n= 3)	3958		3155
SD ±	118		117
p^2			<0.003

Table 12. Summary of the results of immunostaining of normal gastric tissue and primary and metastatic gastric cancers during different stages of tumor progression with mAbs MEM-E/02 and TFL-033a (purified from ascites) and TFL-033s (purified from culture supernatant). (Source: 114]

Tissue Micro Array	Anti-HLA-E-reactive mAbs		Total	MEM-E/02	TFL-033a	TFL-033s
Characteristics	**Incidence**		[n]	%	%	%
			BN 01013a (*Array of Normal Gastric Mucosa***)**			
	normal mucosa		68	*19.1*	**100**	**89.7**
	cell		63	*19*	**100**	**96.8**
			ST 8015 (*Array of Primary tumor tissues***)**			
	Normal tissue in ST 8015		10	*10*	**100**	**100**
	Primary		70	*30*	*37.1*	*38.5*
Types	**Adenocarcinoma**		30	**40**	*26.6*	*30*
	Diffuse carcinoma		40	*22.5*	**45**	**45**
primary tumor infiltration in gastric mucosa*	**T 1 (m, sm)**	**Mucosa/submucosa**	15	*20*	**40**	**46.6**
	T 2 (mp, ss)	**propria/Subserosa**				
	T 3 (se)	Invasion across serosa	55	*32.7*	*36.4*	*36.4*
	T 4 (si)	organ				
Nodal Involvement	**NO**		41	*39*	**51.2**	**53.6**
	YES		29	*17.2*	*17.2*	*17.2*
Stages	**Stage I**	T1/2,N0 or T1,N1	47	*36.2*	**42.6**	**46.8**
	Stage II	T3/4N0				
	Stage III	T4,anyN	23	*17.4*	*26.1*	*21.7*
	Stage IV	anyT,anyN,M1				
Differentiation	Grade 1	Well diff.	21	*42.9*	*38.1*	*38.1*
	Grade 2	moderately diff.				
	Grade 3	**poorly diff**	47	*23.4*	**36.2**	**38.3**
	Grade 4	**not diff.**				
			ST 8013 (*Array of Metastatic tumor tissues***)**			
Metastatic tumor types	**Metastatic lesions**		40	**52.5**	*37.5*	*40*
	peritoneal		5	**40**	*20*	*20*
	Liver		3	*33.3*	*33.3*	*33.3*
	Ovarian		5	**60**	*40*	*40*
	Lymph node		27	**55.6**	*40.4*	*44.4*

Emerging Findings

 # The incidence and Intensity of staining by TFL-033 staining normal gastric mucosa is higher than MEM-E/02

 # The incidence of MEM-E/02 staining Adenocarcinoma is higher than that by TFL-033

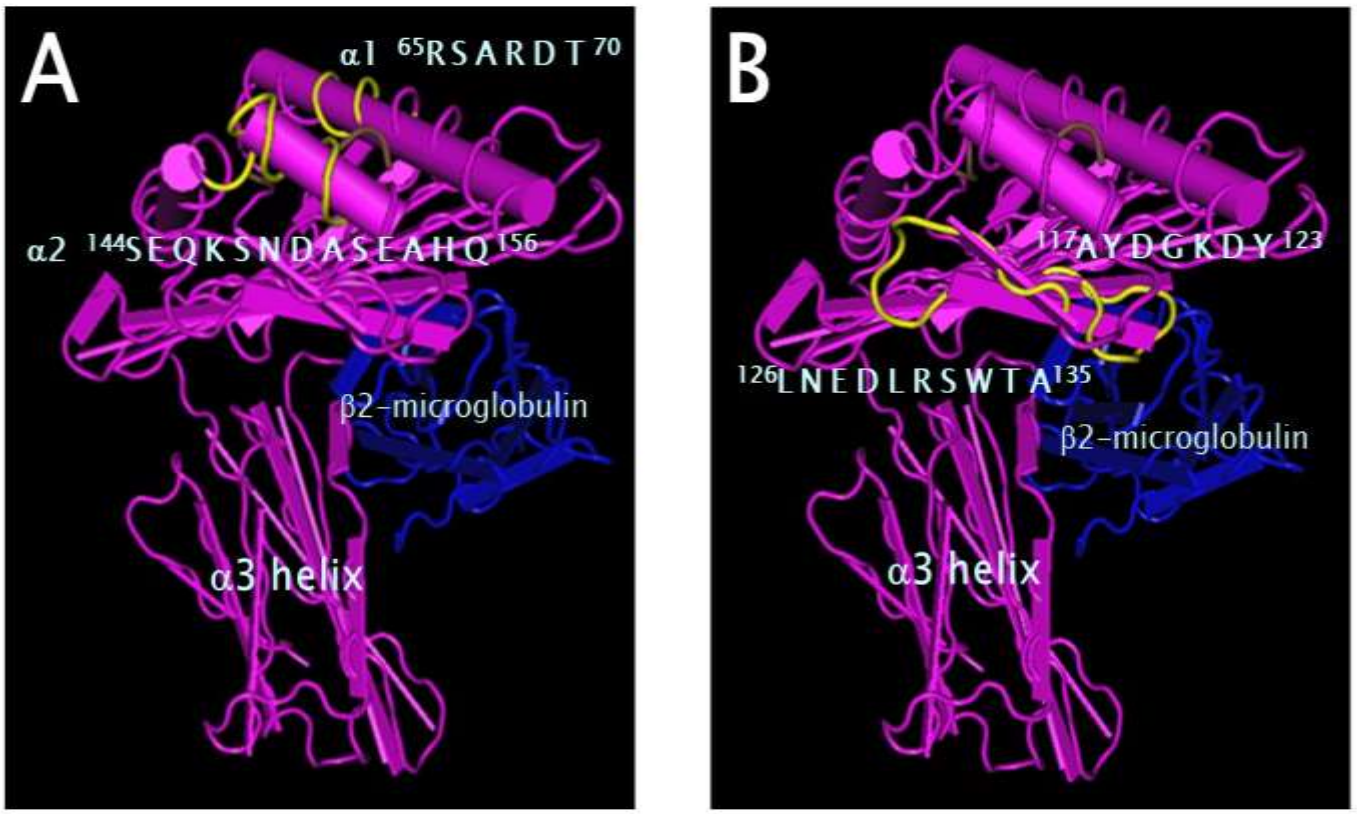
 # The incidence of TFL-033 staining diffuse carcinoma is higher than that by MEM-E/02

 # The incidence of E/02 staining of T3 & T4 is higher than the staining of T1 & T2, no such difference is seen by TFL-033

 # Of all metastatic lesions, the Lymph node lesions stain well with all mAbs in the following order: MEM-E/02 > TFL-033

*** based on** *TNM Classification of Malignant Tumors (UICC)*

Figure 1. The structure of native HLA-E with β2m but without a peptide in the alpha 1 and 2 helical grooves. A. The amino acids specific for alpha 1 (^{65}RSARDT^{70}) and alpha 2 (^{145} SEQKSNDASEAHQ^{156}) helical domains are shown. B. Amino acid sequences common to all HLA class I alleles (^{117}AYDGKDY^{123}, ^{126} LNEDLRSWTA^{135}) are shown.



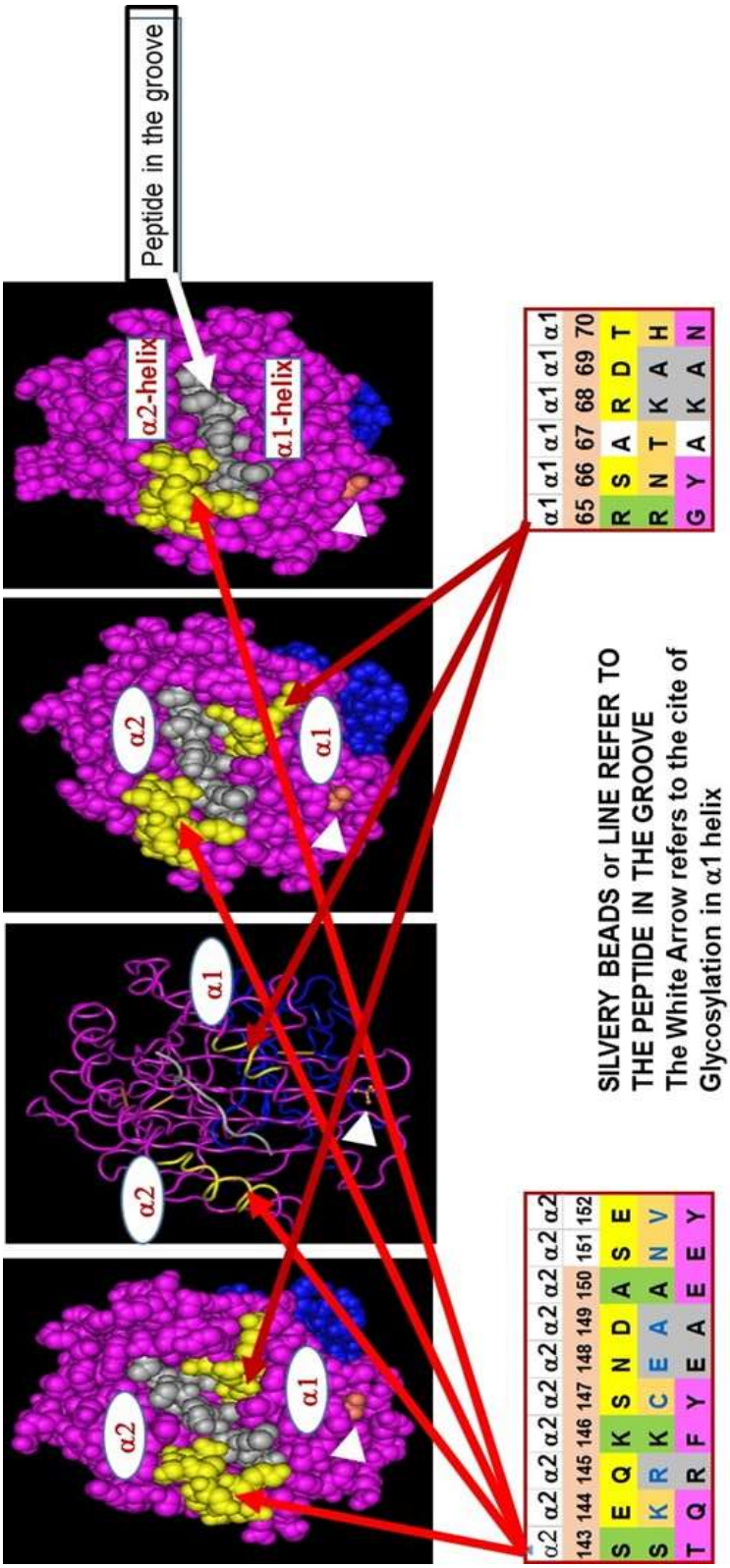


Figure 2. The structure of native trimeric HLA-E with β2m (in blue) and with peptide (silvery beads or line) in alpha 1 and 2 helical groove. The amino acids specific for alpha 1 (^{65}RSARDT^{70}) and alpha 2 (^{145} SEQKSNDASEA^{154}) helical domains are shown. The amino acids in yellow are specific for HLA-E, in orange are specific for HLA-G and in purple for HLA-F. Amino acids common to HLA-E and HLA-G are in green, common to HLA-E and HLA-F are in white, and those common to HLA-G and HLA-F are in Grey.

Figure 3. The structure of native trimeric HLA-E with β2m (in blue) and with peptide (silvery beads or line) in α1 and α2 helical groove. The amino acids specific for α1 (^{65}RSARDT^{70}) & α2 (^{145} SEQKSNDASEA^{154}) helical domains of HLA-E are shown in yellow. Upper three parts of the figure are α2 (^{145} SEQKSNDASEA^{154}) helical domain contains the binding sites of NKG2A of NK cell activating receptors and Lower three parts of the figure are α1 (^{65}RSARDT^{70}) domain cotnains the bindings sites of CD94 of NK cell receptors. The amino acids in orange and in white boxes are specific the amino acids that interact with receptors. Note that these receptors absent in HLA-G and HLA-F.

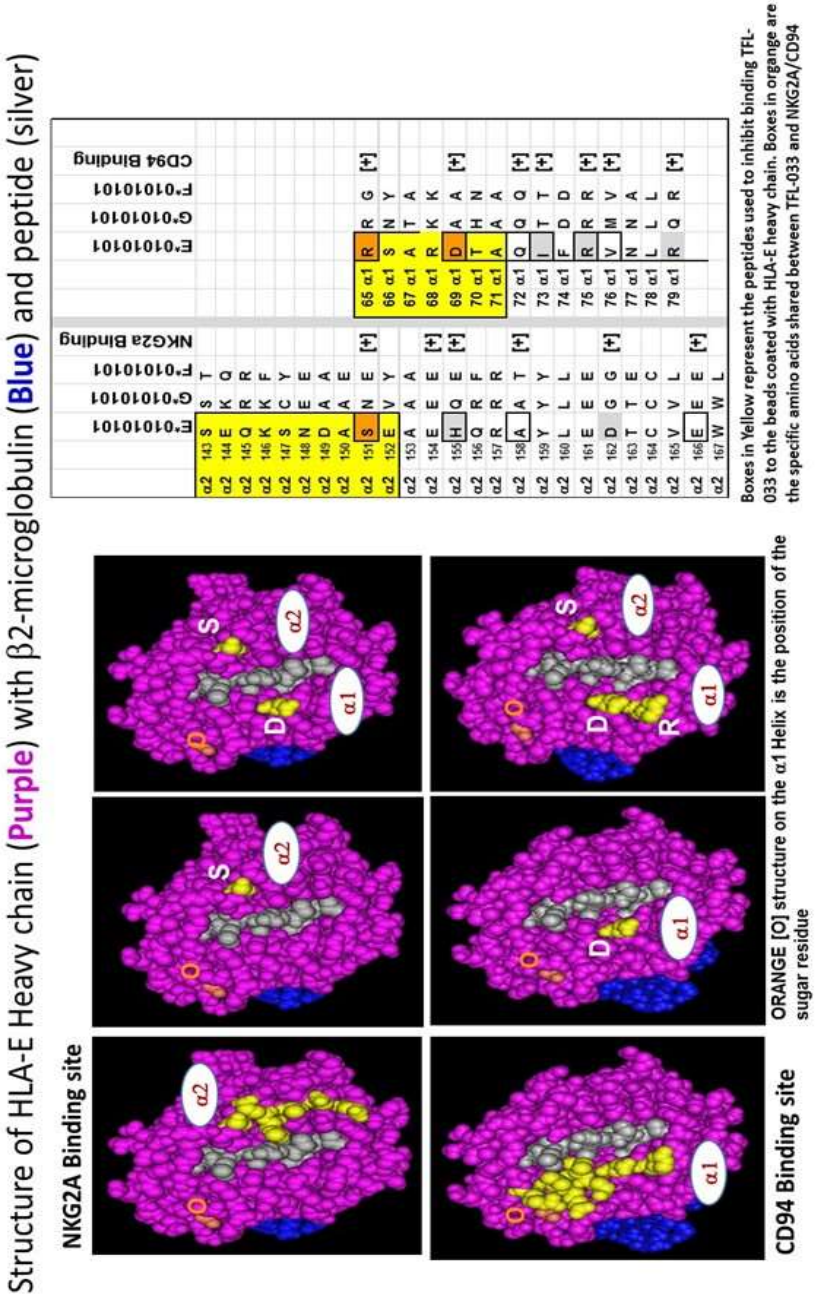


Figure 4. Human gastric cancer (diffused carcinoma) paraffin tissue sections stained with the diluted ascites of monospecificMAb TFL-033a and MEM-E/02. Control, stained without primary MAbs. Note the differences in staining between the two antibodies; MEM-E/02 failed to stain any cells while TFL-033a showed intense and widely distributed staining indicative of overexpression of intact HLA-E [Source: 130].

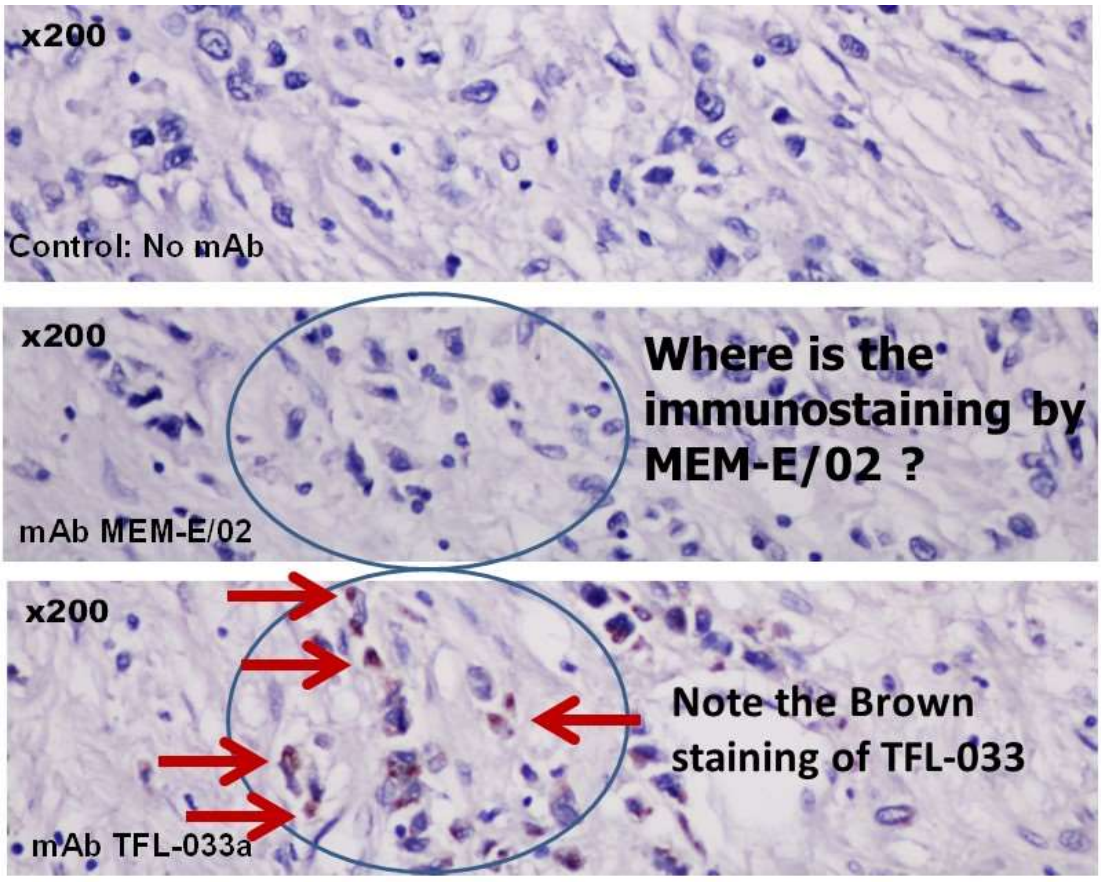
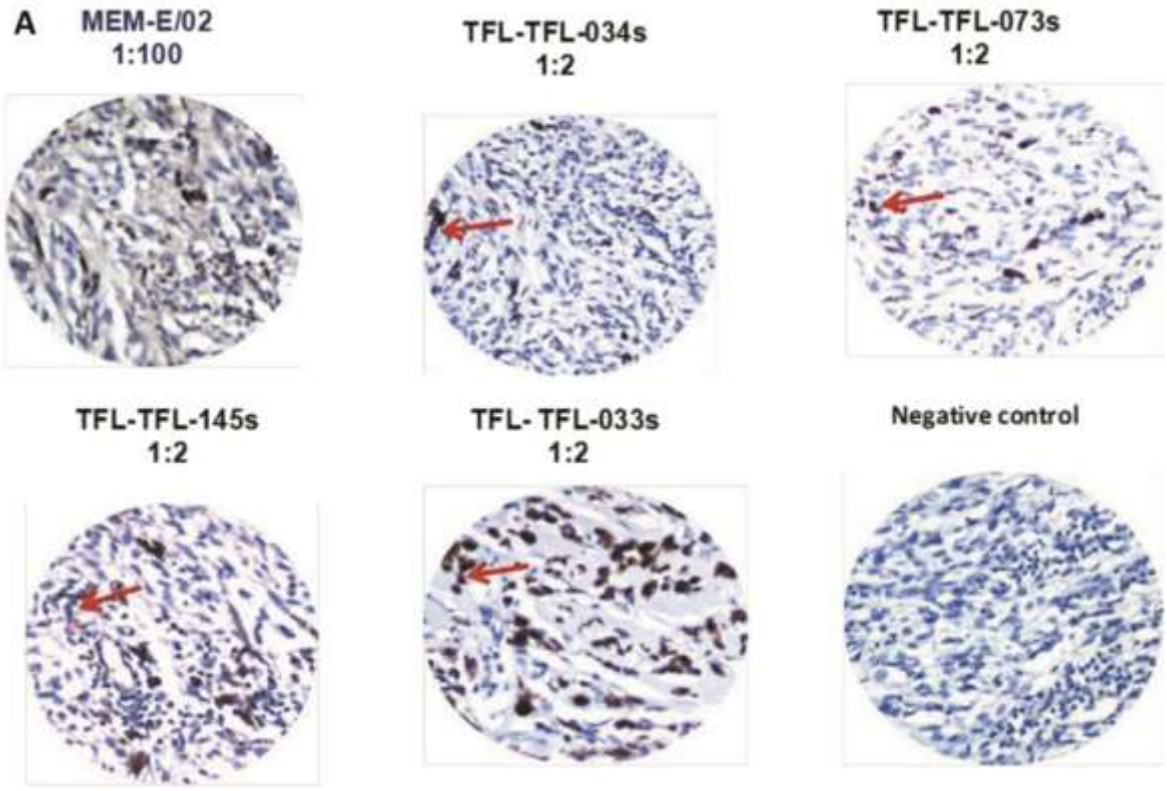


Figure 5. Human melanoma paraffin tissue sections stained with the culture supernatants of TFL monospecific MAbs and of MEM-E/02 [Source: 130]



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