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Stimulation of a T helper cell response against the tumor-associated antigen HER2/neu

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1. Summary

Tumor-reactive $CD4^+$ helper T (Th) cells play a critical role in antitumor immunity. This is due to their ability to induce $CD8^+$ T cell-mediated cytotoxic activity and humoral responses. The herein presented experimental study focuses on the *in vitro* generation and expansion of Th cell lines and clones specific for the tumor-associated antigen human epidermal growth factor receptor-2 (HER2). A protocol for efficient HER2 presentation was developed using autologous monocyte-derived dendritic cells (Mo-DC) as professional antigen presenting cells and purified HER2 protein as the antigen source. In the study it was demonstrated that Mo-DC pulsed with the extracellular domain of HER2 (ECD/HER2) were capable of inducing HER2-specific $CD4^+$ T cell activation. Moreover, the detection of the proinflammatory cytokine IFN-γ by enzyme-linked immunospot analysis and flow cytometry indicated the ability of the antigen-loaded Mo-DC to polarize the T cell response towards a protective Th1 phenotype. This method of immunization can therefore be used for the development of immunotherapy regimens without requiring defined immunogenic epitopes of the antigen.

In an attempt to find optimal conditions for sustaining T cell expansion, long-term culture of the HER2-specific $CD4^+CD8^{lo}$ Th1 cell clone F2.5 with ECD/HER2-loaded autologous Epstein-Barr virus-transformed lymphoblastoid B cells (MaBa-LCL) as stimulator cells promoted the differentiation to a $CD4^-CD8^+$ cytotoxic T cell type. The sequence of phenotypic changes had strong similarities to the CD8 T cell lineage commitment of intermediate $CD4^+CD8^{lo}$ thymocytes as proposed by the kinetic signaling model of T cell development. This gives rise to the suspicion that the precursor cell of the HER2-reactive F2.5 T cell clone had escaped central tolerance and resided in the blood in a dormant state. Under *in vitro* conditions, the self-reactive T cell then became activated by DC-mediated antigenic stimulation and high levels of the immunomodulatory cytokine IL-2.

Based on the assumption of the kinetic signaling model that increased T cell receptor (TCR) engagement promotes CD4 differentiation, whereas weaker signals result in CD8 development, the redirection of the MHC class II-restricted F2.5 T cell clone into the CD8 lineage was suggested to be the consequence of inadequate antigen presentation by MaBa-LCL with decreased expression of stimulatory cytokines such as IL-12 and accessory molecules. In addition, further analysis indicated that two other factors were involved in the process of CD8 conversion. First, antagonist properties of the putative HER2 epitope leading to only partial TCR signal transduction as evidenced by the detection of altered expression levels of the Src family kinases Fyn and Lck. Secondly, exposure to the common cytokine receptor γ chain-dependent prosurvival cytokines IL-2 and IL-7, which are also known to promote the differentiation of intermediate thymocytes into CD8 T cells.

In conclusion, the present study demonstrates that the maintenance of Th1 cell function is inherently dependent on the adequate provision of cytokines, costimulation and TCR engagement. Despite the ability to isolate HER2-reactive T cell clones, the *in vitro* production of sufficient numbers of effector Th1 cells for use in adoptive immunotherapy may be better realized with antigen-specific polyclonal T cell populations.

2. Abbreviations

[^{3}H]TdR, tritiated thymidine; **ABTS**, 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid); **AEC**, 3-amino-9-ethyl carbazole; **APC**, antigen presenting cell(s); **BSA**, bovine serum albumin; **CHO**, chinese hamster ovary; **cpm**, counts per minute; **CTL**, cytotoxic T lymphocyte(s); **DAPI**, 4',6-diamidino-2-phenylindole; **DC**, dendritic cell(s); **EBV**, Epstein-Barr virus; **ECD**, extracellular domain; **EGFR**, epidermal growth factor receptor; **ELISA**, enzyme-linked immunosorbent assay; **ELISPOT**, enzyme-linked immunospot; **ERK**, extracellular signal regulated kinase; **FCS**, fetal calf serum; **FITC**, fluorescein-isothiocyanate; **FPLC**, fast performance liquid chromatography; **HER2**, human epidermal growth factor receptor-2; **HPLC**, high pressure liquid chromatography; **HRP**, horseradish peroxidase; **Ig**, immunoglobulin; **IMAC**, immobilized-metal affinity chromatography; **IU**, international units; **KIR**, killing inhibitory receptor; **KLH**, keyhole limpet hemocyanin; **LAMP**, lysosomal-associated membrane protein; **LCL**, lymphoblastoid cell line; **mAb**, monoclonal antibody; **MAP**, mitogen-activated protein; **MIIC**, MHC class II-rich compartment; **MACS**, magnetic cell sorting; **MHC**, major histocompatibility complex; **MEM**, minimal essential medium; **Mo-DC**, monocyte-derived DC; **MWCO**, molecular weight cut-off; **Ni-NTA**, nickel-nitrilotriacetic acid; **pAb**, polyclonal antibody; **PAGE**, polyacrylamide gel electrophoresis; **PBMC**, peripheral blood mononuclear cell(s); **PBS,** phosphate buffered saline; **PCR**, polymerase chain reaction; **PE**, phycoerythrin; **PGE_2**, prostaglandin E_2; **PI**, propidium iodide; **pMHC**, peptide-MHC; **PNGase F**, peptide N-glycosidase F; **PVDF**, polyvinylidene fluoride; **SD**, standard deviation; **SDS**, sodium dodecylsulfate; **TAA**, tumor-associated antigen; **TCM**, T cell medium; **TCR**, T cell receptor.

3. Introduction

3.1. Adoptive T cell transfer represents a feasible strategy for antitumor treatment

In animal models, established solid tumors can be eradicated by the infusion of large numbers of tumor-specific T cells (Greenberg P.D., 1986; Hanson H.L. et al., 2000; Shu S.Y. and Rosenberg S.A., 1985). Successful use of this approach in animal models has strengthened efforts to apply adoptive T cell immunotherapy also for the treatment of human cancers. This approach appears to be feasible since several clinical studies have demonstrated specific humoral and T cell mediated responses in single cancer patients (Disis M.L. et al., 1994a; Linehan D.C. et al., 1995). Furthermore, preliminary clinical trials in patients with Hodgkin's disease, immunoblastic lymphoma and malignant melanoma provide proof of principle that administration of *ex vivo* expanded T cells may reconstitute effective long lasting immunity *in vivo* (Rooney C.M. et al., 1995; Roskrow M.A. et al., 1998; Yee C. et al., 2000).

3.2. T helper cells are important in the induction and maintenance of cell-mediated immune responses against tumors

It was shown by different authors that successful antitumor treatment depends on the reconstitution of full T cell immunity representing both major T cell subsets, cytotoxic T (Tc) and helper T (Th) cells (Yee C. et al., 2000; Fallarino F. et al., 2000; Huang H. et al., 2002). One of the key features that distinguish the two T cell subsets being their differential coreceptor expression. For Th cells it is the CD4 receptor and for Tc cells the CD8 receptor that initiates the selected T cell function by their respective interaction with major histocompatibility complex (MHC) class II or class I. Th cells are further subdivided into the Th1 and Th2 subsets on the basis of the kinds of cytokines they produce (Kim J. et al., 1985; Mosmann T.R. et al., 1986; Romagnani S., 1995). Many studies have portrayed that Th1 cells are associated with cell-mediated immunity, whereas Th2 cells induce humoral responses (Killar L. et al., 1987; Cher D. and Mosmann T., 1987; Coffman R.L. et al., 1988; Stout R. and Bottomly K., 1989). Thus, it is particularly the Th1 immune response that may contribute to antitumor activity (Nishimura T. et al., 2000). Whereas Tc cells are the main effectors for tumor eradication, Th1 cells are required for the differentiation of naive CD8 T cells into cytolytic effectors.

Helper T cell function is most effective when both Th- and Tc-defined tumor antigen determinants are recognized on the surface of the same antigen presenting cell (APC) (Cassell D. and Forman J., 1988; Bennett S.R. et al., 1997). This phenomenon is attributed to the activity of Th1 cells to engage the APC, which then becomes competent at providing the necessary signals to activate naive Tc cells (Ridge J.P. et al., 1998). Since most tumors express MHC class I but not

class II, the ability of activated APC to induce cytotoxic T lymphocyte (CTL) responses provides the basis for efficient killing of tumor cells (Ossendorp F. et al., 1998).

Once the immune response has been established, Th1 cells can directly support $CD8^+$ Tc cells through secretion of stimulatory cytokines and provision of membrane bound helper functions (Marzo A.L. et al., 2000; Gao F.G. et al., 2002). Most important with respect to antitumor efficacy is that Th1 cells show profound effects on the persistence of CD8 T cell responses. This is most clearly demonstrated by Knutson K.L. et al. (2001), who found that in late stage cancer patients coinfusion of Th cells, specific for a HER2-derived peptide, markedly prolong the life of HER2-reactive CTL.

In addition, it has been observed that in some cases antigen-specific $CD4^+$ Th cells eliminate cancer cells (Greenberg P.D. et al., 1985; Mumberg D. et al., 1999; Qin Z. and Blankenstein T., 2000). The specific mechanisms known to be involved in this $CD4^+$ Th cell-mediated cytotoxicity are induction of apoptosis by expression of death factors such as Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), as well as direct cytolysis by perforin exocytosis (Stalder T. et al., 1994; Kayagaki N. et al., 1999; Abe K. et al., 2000; Vergelli M. et al., 1997).

3.3. Generation of a protective antitumor immune response requires antigen presentation by dendritic cells

The therapeutic goal of eradicating tumors in humans led to the improvement of culture conditions for the efficient generation and expansion of tumor-reactive effector T cells *in vitro*. Several promising strategies of *ex vivo* T cell expansion use dendritic cells (DC) because these are the only APC able to initiate primary T cell responses (Steinman R.M., 1991). Unlike tumor cells, DC express MHC class I and class II molecules as well as various costimulatory and accessory molecules at high levels. Due to this only a minute amount of DC are required to provoke a strong antigen-specific T cell response.

DC undergo two functionally and phenotypically distinct stages. In the immature stage, DC have the capacity to capture foreign antigens by phagocytosis, macropinocytosis or via interaction with C-type lectin and Fcγ receptors (Sallusto F. et al., 1995; Engering A.J. et al., 1997; Jiang W. et al., 1995; Lanzavecchia A., 1996). After lysosomal degradation of endocytosed material DC are able to direct the respective peptide products to specialized MHC class II-rich compartments (MIIC) leading to the formation of large amounts of peptide-MHC (pMHC) class II complexes (Sallusto F. et al., 1995). Alternatively, they can deliver internalized antigens to MHC class I molecules and thus are capable of inducing CTL responses in a process that has been referred to as crosspriming (Russo V. et al., 2000; Berard F. et al., 2000). After antigen uptake and exposure to immune or inflammatory signals, immature DC differentiate to a mature phenotype. During the maturation process, DC lose their antigen-capturing properties and concomitantly increase their T cell-stimulatory capacity by presenting abundant pMHC complexes together with various cos-

timulatory molecules.

In recent years it became obvious that the specific pMHC-TCR interaction is not sufficient to fully activate T cells. Engagement of CD28 by the interaction with the B7 family members CD80 and CD86 on the APC is additionally required as a second signal (Koulova L. et al., 1991). Due to the finding that stimulation of naive T cells in an environment deficient of CD28 costimulation leads to unresponsiveness (Harding F.A. et al., 1992; Lenschow D.J. et al., 1996), the initiation of proper T cell responses depends on antigen-presentation by mature DC with high CD80 and CD86 surface levels (Banchereau J. and Steinman R.M., 1998). Moreover, the shift to a Th1 cytokine profile, necessarily for effective antitumor activity, depends on the ability of the DC to secrete the proinflammatory cytokine IL-12 (Macatonia S.E. et al., 1995; Heufler C. et al., 1996). Because Th1 cells in turn stimulate DC for IL-12 production by CD40 ligation, a positive feed-back loop exists which provides the basis for licensing the DC to promote the observed CTL-mediated immune responses against tumor cells (Cella M. et al., 1996; Schoenberger S.P. et al., 1998; Ridge J.P. et al., 1998; Bennett S.R. et al., 1998).

In vivo, antigen-loaded mature DC migrate to secondary lymphoid organs, such as spleen, lymph nodes and Peyer's patches, where they interact with naive T cells (Banchereau J. et al., 2000; Flores-Romo L., 2001). When stimulated with antigen, naive T cells are primed by the DC to proliferate and differentiate into effector cells. The primed T cells reenter the circulation and perform their effector functions after a second antigen challenge (Butcher E.C. and Picker L.J., 1996).

Attempts to use DC as natural adjuvants for T cell expansion has been facilitated by the advent of culture systems to generate them from blood monocytes (Sallusto F. and Lanzavecchia A., 1994; Romani N. et al., 1996) or $CD34^+$ bone marrow cells (Inaba K. et al., 1992; Young J.W. et al., 1995). To date, a diverse array of growth factors and cytokines has been identified to ensure the induction of DC differentiation from these precursors (for review, see Zou G.M. and Tam Y.K., 2002). However, the majority of research and clinical protocols are based on the well-established method of Sallusto F. and Lanzavecchia A. (1994) where immature DC are differentiated from monocytes by culture in GM-CSF and IL-4, followed by maturation with TNF-α. For use in DC-based immunotherapy, these monocyte-derived DC (Mo-DC) can be efficiently pulsed with synthetic peptides or proteins derived from known tumor-associated antigens (TAA) (Thurner B. et al., 1999; Murphy G.P. et al., 1999; Chaux P. et al., 1999). Additionally, the DC can be successfully transfected with tumor-derived RNA, as reported by Heiser A. et al. (2001).

3.4. HER2 is an appealing target for tumor-associated antigen-specific T cell immunotherapy

The HER2 proto-oncogene encodes for a 185 kDa receptor tyrosine kinase with high homology to the epidermal growth factor receptor (EGFR) (Coussens L. et al., 1985). HER2 overexpression has been detected in subsets of a wide range of cancer types, e.g. adenocarcinomas of

breast, ovary, colon and lung (Hynes N.E. and Stern D.F., 1994; Slamon D.J. et al., 1989) and is a marker of poor prognosis (Walker R.A. et al., 1989; Kern J.A. et al., 1990; Fajac A. et al., 1995). In addition, clinical studies have shown that HER2 overexpression affects as much as 30% of women with breast cancer (Slamon D.J. et al., 1987; Pauletti G. et al., 2000). As was discovered in clinical trials, elevated levels of the HER2 receptor accounts for an aggressive nature of the disease (Di Fiore P.P. et al., 1987; Hadziak R.M. et al., 1987) which responds poorly to hormone or chemotherapy (Slamon D. et al., 1987; Leitzel K. et al., 1995; Vargas-Rodig L.M. et al., 1999).

HER2 is an appealing target for immunologic intervention due to several reasons. The HER2 oncogene is selectively amplified and therefore expressed in high levels only in malignant cells (Slamon D.J. and Clark G.M., 1988). Some patients already present a preexisting cellular and humoral response to HER2, indicating the immunogenicity of the molecule (Disis M.L. et al., 1994a; Fisk B. et al., 1997; Kobayashi H. et al., 2000). As it was shown that HER2 overexpression contributes to the malignant phenotype, it can be assumed that HER2$^+$ tumors will not be able to evade anti-HER2 directed T cell responses by immunoselection of antigen-loss variants, as it was observed for the melanoma-associated differentiation antigen Melan-A/MART-1 (Jäger E. et al., 1996). This assumption is supported by a retrospective study of Niehans G.A. et al. (1993) showing that expression of HER2 in patients who died with metastatic breast cancer is stable over time and generally concruent at different metastatic sites.

The detection of HER2-reactive tumor-infiltrating T cells was the main rationale for the development of T cell-based vaccination strategies that target the HER2 antigen. A recent approach in this field was initiated by Disis M.L. et al. (2004) in the treatment of women suffering from HER2$^+$ breast and ovarian cancer. In this clinical study, the authors were able to induce cellular and humoral HER2-specific immunity in more than 80% of the patients by the administration of a HER2 intracellular domain protein-based vaccine. Their results provide strong support for the feasibility of T cell-based techniques of immunotherapy in HER2$^+$ tumor treatment.

3.5. Escape from central tolerance provide an explanation for the existance of potentially autoreactive T cells in the periphery

In recent years it became evident that large numbers of potentially self-reactive T cells exist in the human blood. This fact was first considered by the isolation of insulin- and myelin basic protein (MBP)-specific T cells from healthy subjects (Kitze B. et al., 1988; Naquet P. et al., 1988; Zhang J. et al., 1994). The presence of autoreactive T cells in the periphery is particularly explained by the fact that the induction of central tolerance in the thymus is not an absolute condition (Bouneand C. et al., 2000). Normally, thymic APC expressing peptides derived from endogenous proteins mediate the negative selection that leads to apoptosis of T cells with high-affinity TCR receptors (Pugliese A. et al., 1997; Egwuagu C.E. et al., 1997; Klein L. et al., 2000). However, some autoreactive T cells can escape thymic deletion either because the specific self

epitope is not expressed in the thymus or is only present in relatively low concentrations on the APC mainly due to inefficient processing or low affinity for the MHC (Milich D.R. et al., 1989; Mamula M.J. and Craft J., 1994; Liu G.Y. et al., 1995).

The self-reactive T cells in the periphery are still subject to control. A complex set of regulatory mechanisms have been identified that can suppress the activation of T cells. Among them are the most important: sequestration of T cells in lymphoid organs, induction of clonal anergy or depletion, inhibition by CTLA-4 coligation and downmodulation by regulatory T cells (Anderton S. et al., 1999; Kamradt T. and Mitchison N.A., 2001).

T cell sequestration is based on the homing receptor profile of naive T cells (Mackay C.R., 1993; Butcher E.C. and Picker L.J., 1996). Due to the binding characteristics of these receptors, the cells are unable to enter normal tissues other than lymphoid organs and are thus physically separated from the majority of tissue antigens. Clonal anergy is induced, when T cells are stimulated by antigen without receiving a costimulatory signal (Harding F.A. et al., 1992; Gimmi C.D. et al., 1993). As a consequence, the T cells become refractory to the antigen even if CD28 signals are provided at the time of restimulation. Activation induced cell death occurs in T cells after repeated stimulation of the TCR (Kabelitz D. et al., 1993; Critchfield J.M. et al., 1994). This process is mediated by the ligation of Fas, a molecule belonging to the tumor necrosis factor receptor family (Brunner T. et al., 1995; Ju S.T. et al., 1995). The most relevant example of T cell inhibition involves upregulation of the CD152 receptor after the initial priming event. CD152 competes with CD28 for binding to the B7 family of ligands on the APC and therefore interrupts the costimulatory pathway of T cell activation (Chambers C.A. and Allison J.P., 1999). Regulatory T cells can inhibit other T cells, most likely through the production of inhibitory cytokines such as IL-10 and TGF-β (Chen Y. et al., 1994; Mason D. and Powrie F., 1998).

Besides genetic susceptibility, considerable evidence implicates infection as the main cause of autoimmune diseases (Kohm A.P. et al., 2003). Mechanisms that can lead from infection to autoimmunity include the release of sequestered autoantigens through tissue damage (Miller S.D. et al., 1997), the activation of a large fraction of the T cell population by superantigens (Perron H. et al., 1997), as well as the induction of inflammatory cytokines and costimulatory molecules by microbial products (Kamradt T. et al., 1991; Klinman D.M. et al., 1996; Tough D.F. et al., 1997). Furthermore, it is suggested by some authors, that structural similarity between microbial and self-antigens may contribute to the activation of autoreactive T cells (Fujinami R.S. et al., 1983; Albert L.J. and Inman R.D., 1999).

3.6. Vaccination with tumor-associated self-antigens can break self tolerance

The inability of naive T cells to recognize endogenous tumor associated epitopes due to the barriers of peripheral tolerance can be overcome by *in vitro* culture methodology. Under appropriate culture conditions, successful induction of a primary T cell response will result in the gen-

eration and expansion of potent effector cells. Due to an increased expression of accessory molecules, such as LFA-1 and CD2, these T cells now have a higher affinity and do not require costimulation for their activation (de Waal Malefyt R. et al., 1993; Bachmann M.F. et al., 1997). As a consequence, they can directly interact with antigen-bearing target cells (Mentzer S.J. et al., 1987; Schmits R. et al., 1996).

T cell-mediated autoimmune responses has been readily achieved *in vitro* against a variety of TAA's mostly by peptide specific stimulation, including tyrosinase (Topalian S.L. et al., 1994; Visseren M.J. et al., 1995; Yee C. et al., 1996), Melan-A/MART-1 (Coulie P.G. et al., 1994; Sensi M. et al., 1995), gp100 (Zarour H. et al., 1996; Kirkin A.F. et al., 1999), MAGE (Traversari C. et al., 1992; Chaux P. et al., 1999), NY-ESO (Jäger E. et al., 1998; Zeng G. et al., 2002) and HER2/neu (Disis M.L. et al., 1994b; Fisk B. et al., 1995).

The most impressive example of how *in vitro* generated effector T cells can mediate autoreactive responses *in vivo* is provided by Yee C. et al. (2000). The authors observed the development of inflammatory lesions in a patient with metastatic melanoma after infusion of CTL clones directed against the melanocyte-specific antigen MART-1. Thus, the results of this study indicate that the adoptive transfer of *in vitro* induced and expanded tumor antigen-specific T cells can break tolerance to generate effective antitumor immunity.

Although the results of recent clinical trials with T cell vaccines are promising, it has been noted by Knutson K.L. et al. (2002) that *ex vivo* expansion methods have to be further optimized to maximise the activity of self-reactive T cells to a level where they can break tumor tolerance. However, as mentioned by the authors, it is of equal interest to tightly control the specificity and the intensity of the immune response in order to avoid the risk of life-threatening hypersensitivity reactions and autoimmune disorders in the cancer patients. Therefore, the objective of T cell-based immunotherapies should be the establishment of a specific and sustained antitumor response while maintaining integrity of the total immune system.

3.7. Purpose of this study

Attempts to treat patients with HER2-overexpressing tumors by adoptive transfer of HER2-reactive T cells have been limited due to the difficulty of generating autologous Th1 cells directed against the HER2 antigen. Thus, the aim of this work was to develop a protocol for the *in vitro* induction of a Th1 cell immunity to HER2. For this purpose, a T cell priming model was established using Mo-DC as APC and highly purified recombinant HER2 protein as the antigen source.

4. Material and Methods

4.1. Cell lines and culture

Dihydrofolate reductase-deficient chinese hamster ovary (CHO) cells transfected with a methotrexate selectable expression vector containing the amino-terminally hexahistidine tagged cDNA coding for the extracellular domain (ECD) of the EGFR (ECD/EGFR) or of the HER2 receptor (ECD/HER2) were a gift from P. Kufer (Ludwig Maximilians-Universität, München, Germany). The cells were initially cultivated in α-minimal essential medium (α-MEM) (Gibco BRL, Karlsruhe, Germany), supplemented with 10% heat-inactivated dialyzed fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany), 100 IU/ml penicillin, 100 IU/ml streptomycin, 2 mM L-glutamine (all from Gibco BRL) and 100 nM methotrexate (Medac, Hamburg, Germany), and then transferred into the protein-free Rencyte K1-medium (MediCult, Jyllinge, Denmark), supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin and 100 nM methotrexate. After an adaptation phase in T_{120}-flasks (Greiner, Nürtingen, Germany) the transfected CHO cells were cultivated to high densities (1-2 x 10^8 cells) in the miniPERM® bioreactor (Vivascience, Hannover, Germany).

Wild-type and HER2 transfected NIH3T3 (NIH3T3^{wt} and NIH3T3^{HER2}) fibroblasts, as well as hybridoma cell lines secreting the mAb's W6/32 and HB55 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in DMEM (Gibco BRL) enriched with 10% FCS, 2 mM L-glutamine and antibiotics.

HER2-negative lymphoblastoid cell lines (LCL) were generated from the donors by Epstein-Barr virus (EBV)-transformation of B cells in peripheral blood mononuclear cell (PBMC) cultures using virus-containing culture supernatant of the marmoset cell line B95-8 (ATCC, Rockville, MD, USA). The MHC class I haplotypes of the cells were characterized by E. Albert (Ludwig Maximilians-Universität, München, Germany) and are presented in table 1. The LCL cells were grown in RPMI 1640 medium (Life Technologies, Paisley, England) supplemented with 10% FCS, 2 mM L-glutamine and antibiotics.

4.2. Immunoblotting

For Western blot analysis, purified protein samples or extract from lysed cells were mixed with an equal volume of sodium dodecylsulfate (SDS) loading buffer consisting of 100 mM Tris-HCl, pH 7.5, 2% SDS and 20 mM dithiotreitol, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions according to the method of Laemmli U.K. (1970). The resolved proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane in buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. The membrane was blocked by over-

Table 1: MHC class I haplotypes of LCL lines

LCL line	HLA-A	HLA-B	HLA-C
MaBa	A3/A68	B35	Cw4/Cw15
JB	A2/A3	B35/B62	Cw4
NvB	A2/A24	B39/B44	Cw5/Cw7
ClMu	A3/A28	B7/B62	Cw3

night incubation at 4°C with 5% skim milk (Fluka, Taufkirchen, Germany) in TBST buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). Transferred antigens were stained with 0.5 to 5 µg/ml mAb in 5% skim milk-TBST for 2 h at RT. After extensive washing with TBST, the membrane was incubated with 20 to 50 ng/ml of horseradish peroxidase (HRP)-conjugated isotype-matched secondary immunoglobulin (Ig) for 1 h at RT. Peroxidase activity was either visualized directly on the blot using 3-amino-9-ethyl carbazole (AEC) (Sigma, Deisenhofen, Germany) as a chromogen or revealed by enhanced chemiluminescence (Pierce, Bonn, Germany) and autoradiography using Hyperfilm® (Amersham Pharmacia Biotech, Freiburg, Germany).

4.3. Purification of recombinant proteins and preparation of cell lysates

Secreted ECD/EGFR and ECD/HER2 protein was purified from CHO cell culture supernantant by immobilized-metal affinity chromatography (IMAC). To improve binding to the nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) interfering reagents of the cell culture supernatant were removed by dialysis against phosphate buffered saline (PBS) using the crossflow-filtration modules Vivaflow-50® with a molecular weight cut-off (MWCO) of 100 kDa (Vivascience). Subsequent IMAC purification was carried out at RT on an ÄktaFPLC® system (Amersham Pharmacia Biotech). In brief, the hexahistidine tagged proteins were incubated overnight at RT under continuous rotation with the nickel resin, loaded onto a HR10/10-column (Amersham Pharmacia Biotech) and connected to the FPLC flow system. After extensive washing with PBS containing 20 mM imidazole (Merck, Darmstadt, Germany) at a flow rate of 0.5 ml/min, recombinant proteins were eluted by increasing the imidazole concentration to 80 mM. The collected peak fraction was dialyzed overnight at 4°C against PBS and concentrated using Centricon® centrifugal filter devices with a MWCO of 50 kDa (Millipore, Bedford, MA, USA). Identity of the purified proteins was confirmed by immunoblot analysis with the Penta-His mAb (Qiagen) and HRP-conjugated goat anti-mouse Ig (Amersham Pharmacia Biotech). Purity was proven to be >95% by detection of protein bands with Brillant Blue R (Sigma). Finally, the protein concentration was determined with the DC protein assay® (BioRad, München, Germany) and sterile filtered aliquots were stored at -80°C.

Recombinant ECD/HER2 produced in a mouse L cell expression system was a gift from T.

Vedvick (Corixa, Seattle, WA, USA). The protein is identical to ECD/HER2 derived from transfected CHO cells except for the absence of a polyhistidine tag and was purified from the cell culture supernatant by ConA sepharose and anion exchange chromatography. The protein preparation was proven to be of high purity in reverse phase HPLC and gel electrophoresis (T. Vedvick, personal communication).

Keyhole limpet hemocyanin (KLH) was purchased in a soluble form from Sigma. NIH3T3^{wt} and NIH3T3^{HER2} cell lysates were prepared by resuspending 1 x 10^6 cells in 200 µl bidistilled water followed by three cycles of rapid freeze-thawing.

For W6/32 and HB55 mAb purification, 0.45 µm-filtered supernatant of the hybridoma cultures was dialyzed against PBS using Vivaflow-50 modules with a MWCO of 10 kDa. After binding to Protein A sepharose (Amersham Pharmacia Biotech) and extensive washing with PBS, antibodies were eluted with an acidic buffer consisting of 100 mM glycine, pH 2.5, and 150 mM NaCl. The pH-value of the eluted peak fraction was immediately adjusted to neutral with 20 mM Tris-HCl, pH 9.0. Subsequently, the antibody preparation was dialyzed overnight at 4°C versus PBS, and after purity control by SDS-PAGE and Coomassie blue staining sterilely filtered and stored in aliquots at -80°C.

4.4. PNGase F-digestion of ECD/HER2

For the detection of N-linked glycans, 10 µg purified ECD/HER2 protein from transfected CHO and L cells was dissolved to 0.5 µg/ml in PBS. 25 units peptide N-glycosidase F (PNGase F) (New England Biolabs, Frankfurt am Main, Germany) were added and digestion was carried out for 1 h at 37°C in a waterbath under continuous shaking. Then the reaction was stopped with 4x reducing sample buffer (Roth, Karlsruhe, Germany) and a total of 0.25 µg of digested ECD/HER2 protein was resolved together with the same amount of unprocessed protein on a 7.5% SDS-PAGE. For Western blot analysis, proteins were transferred to a PVDF membrane and immunostained with a mixture of the mouse mAb's L87 and 2ERB19 (Lab Vision, Newmarket Suffolk, England), both specific for the extracellular domain of HER2, and HRP-conjugated donkey anti-mouse Ig (Santa Cruz Biotechnology, Heidelberg, Germany). Peroxidase activity was then revealed by enhanced chemiluminescence.

4.5. Flow cytometric analysis

Using standard protocols, cells were stained with antibodies in ice-cold PBS containing 0.5% bovine serum albumin (BSA).

For phenotypic analysis of the *in vitro* generated Mo-DC the following fluorescein-isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies were used: FITC-anti-HLA-DR

(Immu-357, mouse IgG1) (Beckman Coulter, Krefeld, Germany), FITC-anti-CD80 (L307.4, mouse IgG1), FITC-anti-CD86 (FUN-1, mouse IgG1), PE-anti-CD83 (HB15e, mouse IgG1), FITC-anti-CD14 (M5E2, mouse IgG2a) and PE-anti-CD54 (HA58, mouse IgG1) (all from BD PharMingen, Heidelberg, Germany). The PE- or FITC-conjugated mAb 679.1Mc7 (mouse IgG1) and the FITC-conjugated mAb U7.27 (mouse IgG2a) (all from Beckman Coulter) were used as isotype controls. Expression of CD40 by immature Mo-DC loaded with soluble ECD/HER2 or NIH3T3^{HER2} cell lysate was evaluated with the FITC-anti-CD40 mAb 5C3 (mouse IgG1) (BD PharMingen). Prior to immunostaining, Mo-DC were incubated for 30 min at 4°C with 20 µl FcR blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany) per 1 x 10^7 total cells to avoid nonspecific binding of antibodies to Fc-receptors.

For quantitative analysis of the TCR Vβ repertoire in the T cell line F2 the Beta Mark® kit from Beckman Coulter was used. Sample preparation was performed according to the manufacturer's instructions. Cells were counterstained with the PECy5-anti-CD4 mAb 13B8.2 or the PECy5-anti-CD8 mAb B9.11, respectively. For the detection of the CD45 isoform on the surface of F2 T cells the FITC-anti-CD45RA mAb HI100 (mouse IgG2b) and the FITC-anti-CD45RO mAb UCHL1 (mouse IgG2a) (both from BD PharMingen) were used. The phenotypic profile of the F2.5 T cell clone isoforms $4^+/8^{lo}$, $4^+/8^+$ and $4^-/8^{hi}$ was assessed by staining with the following mAb's: PE-anti-TCR Vβ5.3 (3D11, mouse IgG1), FITC-anti-CD4 (13B8.2, mouse IgG1), FITC-anti-CD8 (B9.11, mouse IgG1), PE-anti-CD8β (2ST8.5H7, mouse IgG2a), FITC-anti-CD16 (3G8, mouse IgG1) (all from Beckman Coulter), FITC-anti-CD5 (UCHT2, mouse IgG1), FITC-anti-CD27 (M-T271, mouse IgG1), FITC-anti-CD28 (CD28.2, mouse IgG1), PE-anti-CD152 (BNI3, mouse IgG2a) and FITC-anti-HLA-DR (TÜ36, mouse IgG2b) (all from BD PharMingen). KIR expression of the F2.5 T cell clone isoforms $4^+/8^+$ and $4^-/8^{hi}$ was determined with the FITC-anti-NKB1 mAb DX9 (mouse IgG1), the FITC-anti-CD94 mAb HP-3D9 (mouse IgG1), the FITC-anti-NKAT2 mAb DX27 (mouse IgG2a), the FITC-anti-CD158b mAb CH-L (mouse IgG2b) (all from BD PharMingen), the PE-anti-CD158a mAb EB6 (mouse IgG1) and the PE-anti-NKG2A mAb Z199 (mouse IgG2b) (both from Beckman Coulter). Conjugated isotype-matched mAb's (from BD PharMingen or Beckman Coulter) were used as controls in all flow cytometric analyses.

HER2-expression of NIH3T3^{wt} and NIH3T3^{HER2} fibroblasts was determined in a two-step procedure with the mouse anti-human HER2 mAb TA-1 (Calbiochem, Schwalbach, Germany) and FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (Zymed Laboratories, San Francisco, CA, USA).

For exclusion of dead cells, propidium iodide (PI) from Sigma was added to all samples at a final concentration of 0.5 µg/ml just prior to aquisition.

All fluorescence analyses were performed on an Epics Elite ESP flow cytometer (Coulter Electronics, Hialeah, FL, USA). For data evaluation and presentation the WinMDI v2.8 software by Joe Trotter (Scripps Research Institute, La Jolla, CA, USA) and the FlowJo® software (Tree Star Inc., San Carlos, CA, USA) were used.

4.6. Generation of protein-loaded APC

Mo-DC were generated based on a method described by Jonuleit H. et al. (1997). Briefly, PBMC from donor MaBa were isolated by Ficoll® density gradient centrifugation. Monocytes were isolated by plastic adherence and cultured in 6-well plates (Greiner) in X-VIVO-15 (BioWhittaker, Walkersville, MD, USA) supplemented with 1% heat-inactivated autologous plasma, 2 mM L-glutamine, 1600 IU/ml GM-CSF (Sandoz, Nürnberg, Germany) and 1000 IU/ml IL-4 (Strathmann-Biotech, Hannover, Germany). On day 7, the non-adherent Mo-DC precursor were rinsed off, washed once with medium and transferred to fresh 6-well plates at 5 x 10^5 cells in 3 ml medium per well. Subsequent to the addition of 75 µg of purified ECD/HER2 protein, Mo-DC maturation was induced with 1 µg/ml prostaglandin E_2 (PGE_2) (Pharmacia & Upjohn, Kalamazoo, MI, USA) and the following proinflammatory cytokines: 10 ng/ml TNF-α, 10 ng/ml IL-1β, 1000 IU/ml IL-6 (all from Strathmann-Biotech), 500 IU/ml IL-4 and 800 IU/ml GM-CSF. The ECD/HER2-loaded mature Mo-DC were exclusively used to induce a HER2-specific response of naive $CD4^+$ T cells. For restimulation and functional testing purposes Mo-DC of immature phenotype were used instead. These cells were loaded with ECD/HER2 antigen, control proteins or cell lysate and cultivated in X-VIVO-15 medium supplemented with 1600 IU/ml GM-CSF and 1000 IU/ml IL-4 as the only cytokines. On day 10, mature or immature Mo-DC were harvested and used as APC.

Long-term LCL cultures from donor MaBa (MaBa-LCL) were harvested and seeded in U-bottom 96-well plates (Greiner) in a cell number of 5 x 10^5 per well. After addition of 5 µg recombinant protein and 20 h of incubation, cells were harvested, γ-irradiated with 80 Gy and used as APC for T cell expansion or as target cells in functional assays.

4.7. Analysis of antigen processing by Mo-DC

For analysis of antigen processing by immature Mo-DC, 5 mg of ECD/EGFR protein were labeled with a 50 molar excess of the fluorescein-hydroxysuccinimide ester FLUOS (Roche, Mannheim, Germany). After 2 h of incubation at RT, the protein was separated from unbound dye by size-exclusion using a G 25-Sepharose column (Roche). Mo-DC were loaded with the fluorescein-labeled protein at 37°C for various times (30 min, 2 h, 4 h, 8 h and 36 h) and transferred on poly-lysine (Sigma) coated Lab-Tek® chamber slides (Nalge Nunc, Naperville, IL, USA) at a density of 5 x 10^4 cells per chamber. The cells were fixed on the slides with 2% paraformaldehyde (Sigma) for 10 min at RT and permeabilised with methanol for 2 min. After blocking in PBS with 10% goat serum (Sigma) for 1 h at RT, the slides were incubated overnight at 4°C in PBS with 10% goat serum and 20 µg/ml of either the CD206-specific mAb 19, the LAMP-1-specific mAb H4A3 or the HLA-DR-specific mAb TÜ36 (all from BD PharMingen). Subsequently, cells

were exposed to a 1:200-dilution of Texas Red-conjugated goat anti-mouse Ig (Dianova, Hamburg, Germany) in PBS with 10% goat serum for 1 h at RT. Following a 5 min-incubation in PBS containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma) at RT to stain nuclei, the slides were mounted using the Slow Fade Antifade Kit® (Molecular Probes, Eugene, OR, USA). For visualization, a Zeiss Axioskop microscope (Zeiss, Göttingen, Germany) was used with appropriate filters.

4.8. Isolation and expansion of a HER2-specific Th1 cell clone

$CD4^+$ T cells were purified from PBMC of donor MaBa by magnetic cell sorting (MACS) (Miltenyi Biotec). Subsequently, 1 x 10^6 $CD4^+$ T cells were cultured with 2 x 10^4 autologous ECD/HER2-pulsed mature Mo-DC in the wells of 24-well plates (Greiner) in RPMI 1640 supplemented with 5% autologous serum, 2 mM L-glutamine and antibiotics (hereafter referred to as TCM). Following three cycles of weekly restimulations (on day 7, 14 and 21) with autologous ECD/HER2-loaded immature Mo-DC and culture in TCM containing 20 IU/ml IL-2 (R & D Systems, Wiesbaden, Germany) and 5 ng/ml IL-7 (BD PharMingen), each individual T cell culture was analyzed for ECD/HER2-specific IFN-γ release using an enzyme-linked immunospot (ELISPOT) assay (see Section 4.9.1). Activated T cells of the microculture F2, showing the optimum HER2-specific response, were enriched by MACS using an IFN-γ secretion assay (described in Section 4.9.2) and anti-PE paramagnetic beads (both from Miltenyi Biotec). The sorted polyclonal T cells, referred to as T cell line F2, were further expanded. To provide factors for optimal proliferation, 5% T-Stim (BD PharMingen) was additionally added to the TCM in all subsequent cultivation steps. After further evidence for HER2-specificity by IFN-γ ELISPOT and IFN-γ secretion testing, the relative frequency of TCR Vβ motifs in the F2 culture was determined by flow cytometric analysis. TCR $V\beta 5.3^+$ T cells resembling 85% of the $CD4^+$ T cell fraction were enriched by MACS using the PE-labeled anti-TCR Vβ5.3-specific mAb 3D11 and anti-PE paramagnetic beads. Subsequently, sorted T cells were cloned by limiting dilution. Therefore, T cells were plated at 0.3 cells/well in U-bottom 96-well plates with 1 x 10^5 ECD/HER2-pulsed and γ-irradiated MaBa-LCL as APC. The $CD4^+$TCR $V\beta 5.3^+$ T cell clone F2.5, with the best viability and IFN-γ release upon antigen-specific stimulation, was further expanded to high cell numbers by culture in T_{30}-flasks (Greiner) in the presence of 15 x 10^6 ECD/HER2-pulsed γ-irradiated MaBa-LCL. Starting from a total of 1 x 10^5 T cells, 18 days of cell culture resulted in 1-2 x 10^7 T cells. To some of the flask-cultures IL-12 (R & D Systems) was added at a final concentration of 0.2 ng/ml. In parallel, F2.5 T cells were expanded in the wells of 24-well plates precoated overnight at 4°C with mAb 3D11 in PBS (200 µl, 5 µg/ml). After 1 week of antibody-induced proliferation, T cells were passaged to uncoated plates for a 1 week-resting period. Subsequently, T cells were used for functional experiments or further rounds of clonal expansion.

4.9. Functional T cell assays

4.9.1. IFN-γ ELISPOT analyses

For the IFN-γ ELISPOT assay, 2 x 10^4 immature Mo-DC, unpulsed or pulsed with different antigens, were coincubated with 1 x 10^3 autologous T cells overnight at 37°C with 5% CO_2 in the wells of 96-well nitrocellulose filter plates (Millipore) that were coated with the mouse anti-human IFN-γ antibody 1-D1K (Mabtech, Nacha, Sweden). After removal of cells by extensive washing with PBS, captured IFN-γ was detected by the sequential addition of the biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech), HRP-conjugated streptavidin (ABC Vectastain-Elite Kit®) (Vector, Burlingame, CA, USA) and the peroxidase substrate AEC, as previously described by Herr W. et al. (1996). Red spots corresponding to single IFN-γ secreting cells were counted by dissecting microscopy (Zeiss).

4.9.2. IFN-γ secretion assays

1 x 10^5 T cells were stimulated with 1 x 10^4 protein-loaded immature Mo-DC in the wells of U-bottom 96-well plates overnight at 37°C with 5% CO_2. For blocking experiments, 10 μg/ml of the mAb's HB55 (anti-HLA-DR), W6/32 (anti-MHC I), RIV II (anti-CD8) or anti-CD4 (anti-CD4) (the latter two from DPC Bierman, Bad Nauheim, Germany) were added 30 min prior to establishment of the coculture either to the Mo-DC (HB55 and W6/32) or to the T cells (RIV II and anti-CD4). Cells were harvested and washed once in PBS supplemented with 0.5% BSA. Subsequently, an anti-IFN-γ x anti-CD45 Fab-trimer construct called IFN-γ catch reagent, was allowed to attach to the cell surface of all T cells by a 10 min-incubation of 20 μl of the reagent per 1 x 10^7 cells at 4°C. After dilution to 1 x 10^5 cells/ml with 37°C warm RPMI 1640 medium, cells were incubated for 45 min at 37°C under slow continuous rotation. In this time period, IFN-γ secreted by an activated T cell is captured locally on the cell surface by the IFN-γ catch reagent. For subsequent fluorometric detection of surface-bound IFN-γ, T cells were stained with a PE-anti-IFN-γ mAb (Miltenyi Biotec). Additionally, cells were labeled with the FITC-anti-CD4 13B8.2 mAb and the PECy5-anti-CD8 B9.11 mAb. For exclusion of dead cells, PI was added prior to aquisition. A lymphocyte gate was set based on forward and side scatter properties. Finally, four-color analyses were performed on an Epics Elite ESP flow cytometer (Coulter Electronics).

4.9.3. ^{51}Cr release assays

CTL activities were measured in a standard ^{51}Cr release assay. In brief, 1 x 10^6 protein-loaded immature Mo-DC were labeled in 100 μl of FCS with 100 μCi/ml ^{51}Cr (ICN Biochemicals, Irvine, CA, USA) for 1.5 h at 37°C. Graded numbers of T cells were given to 1 x 10^4 ^{51}Cr-labeled target

cells in the cavities of a V-bottom 96-well tissue culture plate (Costar, Cambridge, MA, USA). Following incubation for 4 h at 37°C with 5% CO_2, the supernatant was collected and radioactivity was measured in a γ-counter. Specific lysis was calculated as follows: % specific ^{51}Cr release = ((experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(maximum ^{51}Cr release - spontaneous ^{51}Cr release)) x 100. Maximum ^{51}Cr release was obtained by adding 100 µl of 1% Nonidet P-40 (Sigma) to 100 µl of labeled target cells.

4.9.4. Proliferation assays

To assess proliferative capacity of HER2-specific T cells, 1 x 10^4 T cells were incubated in 200 µl TCM with 1 x 10^3 unpulsed or protein-loaded γ-irradiated MaBa-LCL in U-bottom 96-well plates. After 4 d of culture at 37°C with 5% CO_2, tritiated thymidine ([^{3}H]TdR) (Amersham Pharmacia Biotech) was added at 1 µCi/well and incubation was prolonged for additional 16 h. [^{3}H]TdR incorporation was estimated by harvesting cells onto filter plates using a 96-well cell harvester (Packard Instrument, Downers Grove, IL, USA) and counting in a β-scintillation counter (LKB Wallac, Erlangen, Germany).

4.10. Cytokine detection by ELISA

IL-12 p40, IFN-γ and TNF-α were measured in T cell-DC culture supernatants harvested 20 h after T cell stimulation using standard sandwich enzyme-linked immunosorbent assay (ELISA) protocols. Antibodies and recombinant standards of all cytokines were obtained from BD PharMingen. The capture antibody was bound to the ELISA plate (Nunc, Roskilde, Denmark), the biotinylated detection antibody was revealed by HRP-conjugated streptavidin and 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) substrate (Sigma), and the assay read at 415 nm.

4.11. Signal transduction analysis

20 h after initiation of coculture, 1 x 10^6 T cells and 2 x 10^4 antigen-loaded immature Mo-DC were lysed in SDS gel loading buffer. Whole cell extracts were then resolved by 7.5% SDS-PAGE and transferred to a PVDF membrane. Subsequently, the membrane was immunostained with a variety of antigen-specific mAb's and species-matched HRP-linked secondary Ig's. Peroxidase activity was visualized by enhanced chemiluminescence. After each immunodetection, the membrane was stripped of bound antibodies by three 10 min-washings in a mixture of 45% methanol and 10% glacial acid at RT under continuous shaking. Then the membrane was blocked again and exposed to the next antibody.

The membrane was probed in the following order: First, at threonine-202 (Thr-202) and tyrosine-204 (Tyr-204) dually phosphorylated p44 and p42 mitogen-activated protein (MAP) kinases were detected with the mouse anti-human phospho-p44/42 MAPK (Thr202/Tyr204) mAb E10 (New

Table 2: PCR Primers for RT-PCR analysis

PCR Group	Sense Primer (5'-3')	Antisense Primer (5'-3')	Location[a]	Length
β-actin	CCA AGG CCA ACC GCG AGA AGA TGA C	AGG GTA CAT GGT GGT GCC GCC AGA	335 - 922	587
CD4	GCA AGG CCA CAA TGA ACC GG	GCC TGC CAC CAC AGC TCG	-11 - 725	736
CD8α	CGC GTC ATG GCC TTA CC	TGA CCA CAG GCC GGG GAC	-6 - 667/557[b]	673/563[b]
CD8β	CAC GAT GCG GCC GCG	GCA GCA CAG GTG GAT GGC	-4 - 585	589
TCR Vβ5.3	CTG TGC CAT GGG CCC TG	GCT GCT GGC ACA GAG AT	-11 - 339	350
KIR 2DL1	GCA GCA CCA TGT CGC TCT	GTC ACT GGG AGC TGA CAC	-8 - 348	356
KIR 2DL2	CCA CTG CTT GTT TCT GTC AT	CAG CAT TTG GAA GTT CCG C	645 - 1015	370
KIR 2DL3	CCA CTG AAC CAA GCT CCG	CAG GAG ACA ACT TTG GAT CA	692 - 1044	352
KIR 2DL4	CTG TCC CTG AGC TCT ACA A	CAC TGA GTA CCT AAT CAC AG	206 - 747	541
KIR 3DL1	ACA TCG TGG TCA CAG GTC C	TGC GTA TGT CAC CTC CTC	641 - 1197	556
KIR 3DL2	CGG TCC CTT GAT GCC TGT	GAC CAC ACG CAG GGC AG	546 - 914	368
KIR 2DS1	TCT CCA TCA GTC GCA TGA A	AGG GCC CAG AGG AAA GTT	254 - 567	313
KIR 2DS2	TGC ACA GAG AGG GGA AGT A	CAC GCT CTC TCC TGC CAA	179 - 435	256
KIR 2DS3	TCA CTC CCC CTA TCA GTT T	GCA TCT GTA GGT TCC TCC T	315 - 594	279
KIR 2DS4	CTG GCC CTC CCA GGT CA	GGA ATG TTC CGT TGA TGC	94 - 544	450
KIR 2DS5	AGA GAG GGG ACG TTT AAC C	GCC GAA GCA TCT GTA GGC	184 - 600	416
KIR 3DS1	ATA GGT TTA ACA ATT TCA TG	AAG GGC ACG CAT CAT GGA	91 - 501	410

[a] nucleotide positions of amplified fragments were calculated from the start codon
[b] shorter fragment through alternative splicing of mRNA

England Biolabs) and HRP-conjugated donkey anti-mouse Ig. Then total levels of these endogenous extracellular signal regulated kinase (ERK) molecules were determined with the rabbit anti-human p44/42 MAP kinase pAb (New England Biolabs) and HRP-conjugated goat anti-rabbit Ig (Santa Cruz Biotechnology). Next, expression of the Src tyrosine kinase Fyn was evaluated with the goat anti-Fyn pAb FYN3 (Santa Cruz Biotechnology) and HRP-conjugated donkey anti-goat Ig (Amersham Pharmacia). This was followed by staining for Lck, another T cell-specific Src family kinase, using the mouse anti-Lck mAb 3B5 and HRP-conjugated donkey anti-mouse Ig. Finally, the mouse mAb JLA20, an IgM specific for α-, β- and γ-actin, and HRP-conjugated goat anti-mouse IgM pAb (both from Calbiochem) were used to compare the amount of protein in each lane.

4.12. RNA extraction and reverse transcription

For polymerase chain reaction (PCR) typing of killing inhibitory receptor (KIR) molecules, total cellular RNA was prepared from 1 x 10^7 cells. The KIR-specific primers were synthesized based on the sequences given by Uhrberg M. et al. (1997) and are presented in table 2. Reverse transcription and PCR was done in one step using the Titan One Tube RT-PCR Kit® (Roche) according to the manufacturer's instructions. Amplification of the KIR was performed under the same conditions with an annealing temperature of 58°C for all primer sets. For the determination of CD4, CD8α, CD8β and TCR Vβ5.3 mRNA expression, 1 x 10^6 T cells were subjected to RT-PCR using the OneStep RT-PCR Kit® (Qiagen). The sequences of the primers are also listed in table 2. Amplification products were separated together with the β-actin control on 2% agarose gels containing ethidium bromide and visualized on an ultraviolet transilluminator.

5. Results

5.1. CHO cell culture results in high-level production of recombinant protein

CHO cells stably transfected with ECD/EGFR or ECD/HER2 were cultured under serum- and protein-free conditions. Without serum supplement the CHO cells lost their capacity to adhere on the plastic of the culture vessel and grew in suspension forming small spheric cell aggregates. Microscopic examination with trypan blue dye suggested that these cell clusters were almost free of dead cells. Detached CHO cells continued to proliferate even after transfer into the production module of a bimodular minifermenter and incubation in rotary culture. The observed growth rates were similar to those in serum-supplemented cultures suggesting equivalent doubling times. Compared to stationary cultures in flasks or cell factories, much higher cell densities were achieved. Consequently, collected cell culture supernatants contained markedly higher levels of secreted recombinant proteins as judged by protein yields after purification (data not shown).

5.2. A simple two-step purification allows isolation of highly pure ECD/HER2 antigen

The establishment of a high density CHO culture in medium without serum was the prerequisite for obtaining pure recombinant proteins in the subsequent two-step FPLC column purification protocol. Starting with 150 ml of CHO cell culture supernatant, a yield of 0.5-2 mg pure protein was achieved by tangential flow filtration and IMAC purification.

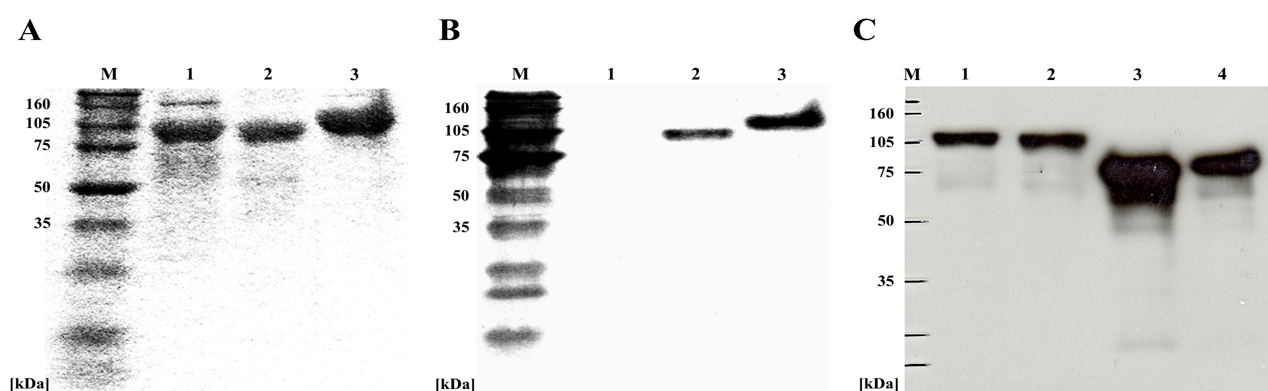

FIGURE 1: Quality control of purified proteins. Detection of the recombinant ErbB proteins after separation in a 7.5% SDS-PAGE (**A**) by Coomassie blue staining and (**B**) by immunoblot analysis with the Penta-His mAb using AEC as chromogen. **Lane 1**, ECD/HER2 from transfected L cells; **lane 2**, ECD/HER2 from transfected CHO cells; **lane 3**, ECD/EGFR from transfected CHO cells. (**C**) Enhanced chemiluminescence detection of purified ECD/HER2 proteins before and after PNGase F-treatment with a combination of the two HER2-specific mAbs L87 and 2ERB19. **Lane 1**, ECD/HER2 from L cells; **lane 2**, ECD/HER2 from CHO cells; **lane 3**, PNGase F-digested ECD/HER2 from L cells; **lane 4**, PNGase F-digested ECD/HER2 from CHO cells. **M**, molecular weight marker.

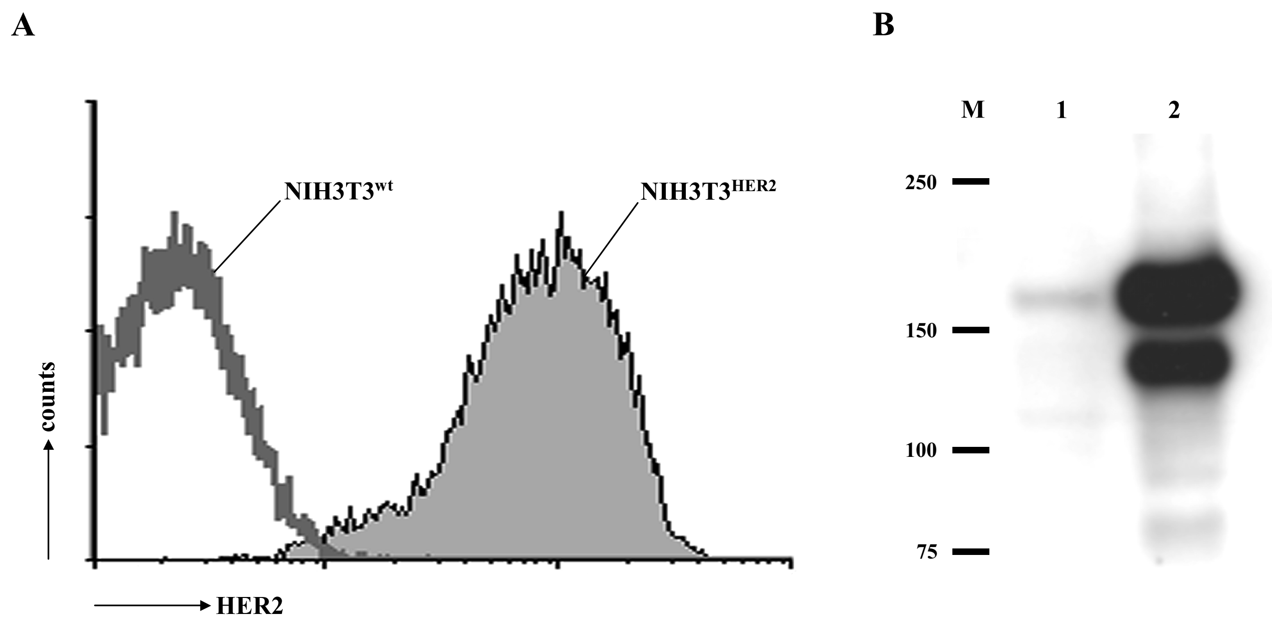

FIGURE 2: Comparison of HER2 expression on native and HER2-transfected NIH3T3 mouse fibroblasts. (**A**) Cells were immunostained in a two-step procedure with the HER2-specific mAb TA-1 and FITC-conjugated F(ab')$_2$ goat anti-mouse Ig and measured by flow cytometry. **Empty curve**, NIH3T3^{wt} cells; **filled curve**, NIH3T3^{HER2} cells. (**B**) After separation of whole protein extracts from 1 x 10^5 cells in a 5% SDS-PAGE and electroblotting onto a PVDF membrane, HER2 expression of NIH3T3^{wt} and NIH3T3^{HER2} fibroblasts was evaluated by immunostaining with the anti-HER2 mAb 3B5, recognizing amino acid residues 1242-1255 of the intracellular kinase domain. **Lane 1**, NIH3T3^{wt} cells; **lane 2**, NIH3T3^{HER2} cells. **M**, molecular weight marker.

The quality of purification was monitored by Coomassie blue staining of SDS-PAGE gels and immunoblot detection of the N-terminal hexahistidine sequence. For comparison, ECD/HER2 produced in a L cell expression system was analyzed on the same gel. As shown in Fig. 1A, Coomassie blue staining revealed a single protein band between the 75 and 105 kDa marker for CHO-derived ECD/HER2 and ECD/EGFR. However, in the ECD/HER2 sample from L cells an additional band with an apparent molecular weight of >160 kDa was observed. This unidentified protein may correspond to a dimeric form of the ECD/HER2 molecule as an artefact of purification. Western blot analysis with the Penta-His mAb identified the proteins between 75 and 105 kDa in lane 3 and 4 of the Coomassie blue stained gel as the expected His-tagged recombinant proteins (Fig. 1B). The specificity of the mAb was confirmed by the absence of immunoreactivity for L cell-derived ECD/HER2.

Both ECD/HER2 preparations were treated with PNGase F and analyzed by immunodetection with mAb's directed against the extracellular domain of the HER2/neu receptor (Fig. 1C). As control, unprocessed ECD/HER2 samples were resolved on the same gel. The observed change in mobility from approximately 100 to 75 kDa in response to PNGase F-treatment indicates that N-linked oligosaccharides contribute substantially to the apparent molecular mass of recombinant ECD/HER2. After removal of glycan residues from the protein backbone, ECD/HER2 displayed the size expected from the cDNA nucleotide sequence, which is 70 kDa.

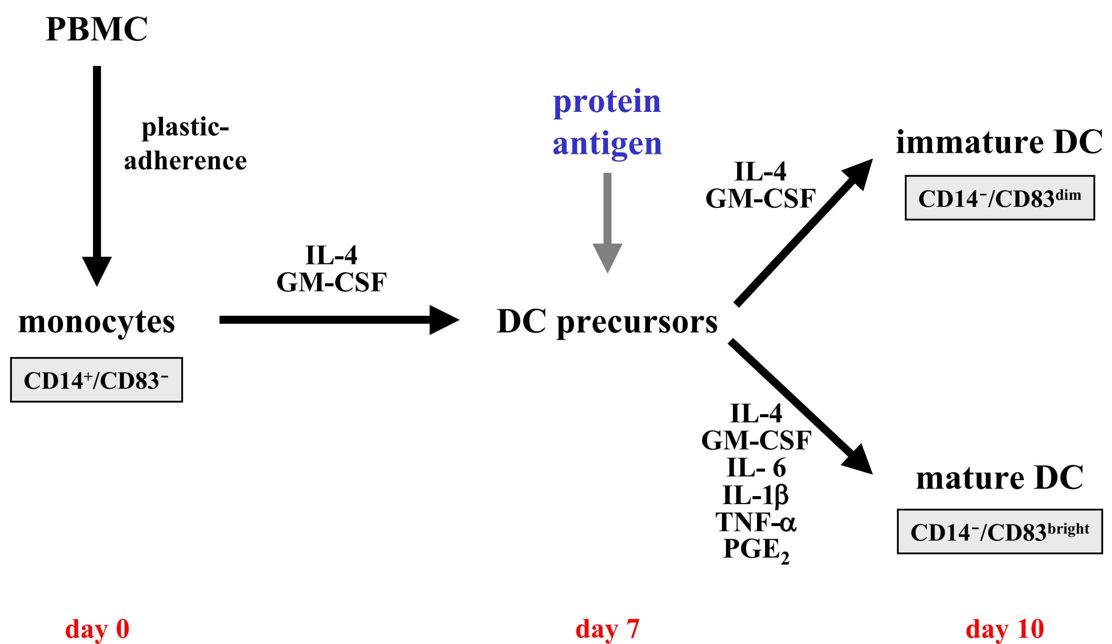

FIGURE 3: Generation of protein-loaded immature and mature DC under FCS-free conditions. Monocytes from PBMC of donor MaBa were isolated by plastic-adherence and differentiated into DC precursors by IL-4 and GM-CSF. At day 7, nonadherent cells were transferred to fresh plates, pulsed with protein antigens and either cultivated with IL-4 and GM-CSF for the generation of immature DC or further matured with a cocktail of the proinflammatory cytokines TNF-α, IL-1β and IL-6 and the prostaglandin PGE_2. **Grey shaded boxes**, CD14 and CD83 phenotype of the cells, as revealed by flow cytometric analysis.

5.3. Whole cell extract from HER2-transfected NIH3T3 fibroblasts provides a second high-quality antigen source

Whole cell lysate from $NIH3T3^{HER2}$ cells was tested for its potential usage as a second HER2 antigen source. As detected by flow cytometry, the intensity of staining for HER2 was approximately 50-fold higher on the HER2-transfectants compared to wild-type NIH3T3 cells (Fig. 2A). In accordance with these findings, immunoblot detection of HER2 with a mAb directed against the intracellular domain of the receptor revealed no expression by $NIH3T3^{wt}$ cells, but a remarkably strong expression by $NIH3T3^{HER2}$ cells (Fig. 2B). Therefore, lysate of $NIH3T3^{HER2}$ cells could serve as a control to prove the specificity of T cells raised against ECD/HER2.

5.4. The *in vitro* generated Mo-DC are highly pure and assume an uniform CD83 phenotype

As outlined in Fig. 3, Mo-DC were generated by culturing adherent cells from PBMC of donor MaBa in the presence of IL-4 and GM-CSF. Autologous plasma was used to avoid loading the Mo-DC with allogeneic proteins. As a result of cytokine treatment, most of the cells detached from the plastic surface and acquired the classical DC morphology with prominent dendrites and abundant veils. Flow cytometric analysis of these non-adherent cells on day 7 confirmed the successful differentiation to the DC phenotype by the absence of the monocyte marker CD14 and the

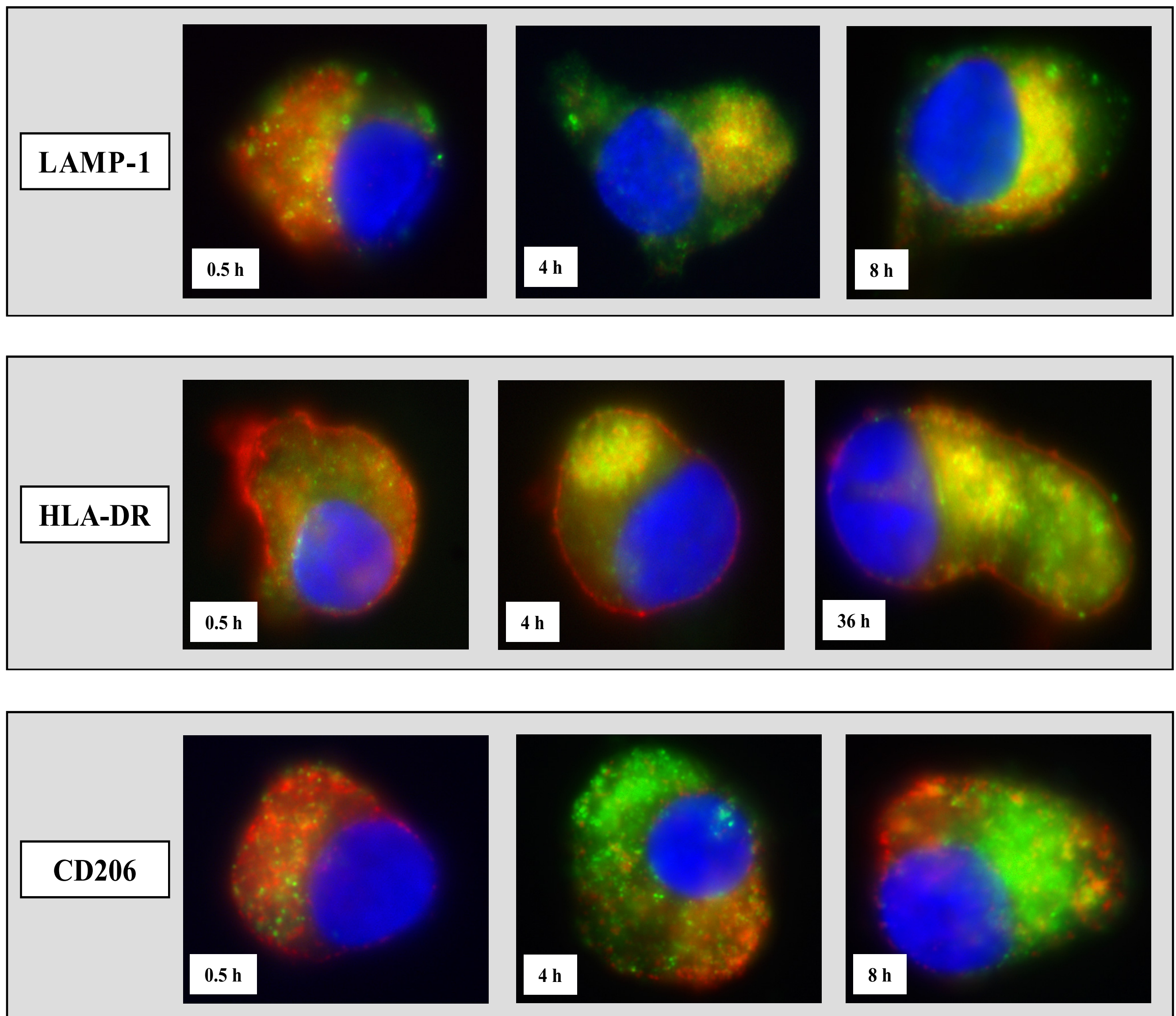

FIGURE 4: Immunofluorescence analysis of ErbB antigen processing in immature Mo-DC. Immature Mo-DC were pulsed with fluorescein-conjugated ECD/EGFR protein (**green**). At the indicated times, DC were transferred to poly-lysine coated slides. After fixation with paraformaldehyde and permeabilization with methanol, DC were immunostained with mouse mAb's specific for the cellular markers LAMP-1, HLA-DR and CD206, followed by Texas Red-labeled goat anti-mouse Ig (**red**). In a color overlay colocalisation of the ingested antigen with the intracellular markers is indicated by the yellow color. Nuclei were stained with DAPI (**blue**).

expression of the DC lineage marker CD83. The purity of these DC cultures was found to be >90% with the major contaminants being B lymphocytes. Due to their high immunostimulatory capacity, ECD/HER2-loaded mature DC, generated by treatment with a combination of proinflammatory cytokines and the prostaglandin PGE_2, were used to prime naive autologous $CD4^+$ T cells. In the subsequent restimulations and functional analyses, the less immunogenic antigen-loaded immature DC were applied to the T cell cultures because, in preliminary experiments, they were found to be sufficient for primed T cell activation and had the advantage not to promote activation-induced T cell death. Discrimination between immature and mature DC was performed on day 10 by flow cytometric detection of CD83 surface levels.

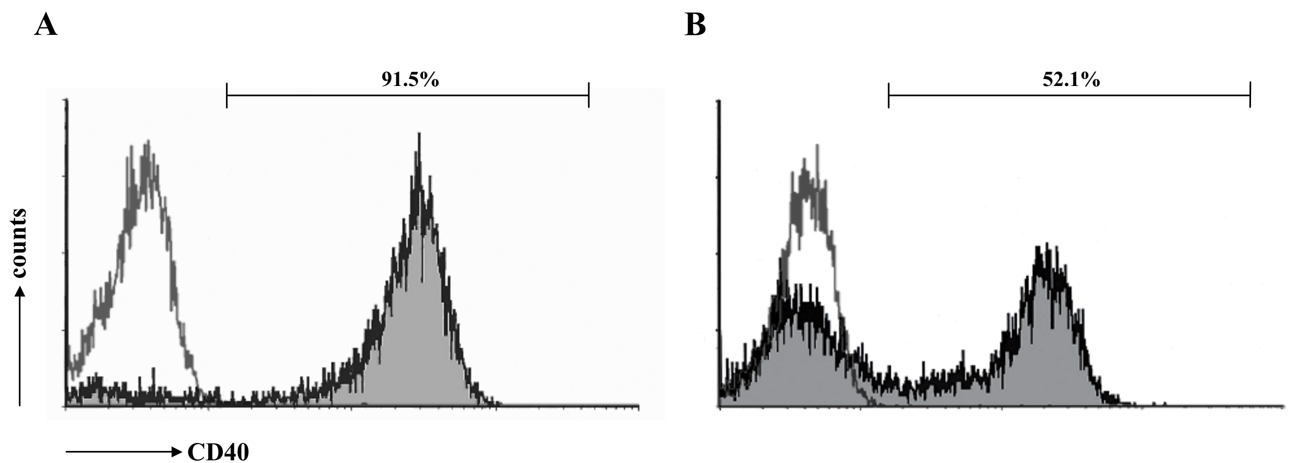

FIGURE 5: Differential expression of CD40 on immature Mo-DC after exposure to ECD/HER2 or NIH3T3^{HER2} cell lysate. 3 days after antigen addition, immature Mo-DC were immunostained with the CD40-specific mAb 5C3 and analyzed by flow cytometry. Histograms of (**A**) ECD/HER2- and (**B**) NIH3T3^{HER2} cell lysate-loaded immature Mo-DC. Empty curves represent the isotype-matched antibody control.

5.5. Immature Mo-DC rapidly take up soluble protein antigens

The endocytotic pathway of ingested ErbB protein antigens was evaluated exemplarily for ECD/EGFR by immunofluorescence microscopy. As shown in Fig. 4, fluorescein-labeled ECD/EGFR was visible in the DC 30 min after antigen addition. 3.5 h later the protein was localized predominantly perinuclear in vesicles resembling lysosomes and MHC class II-rich late endosomes (MIIC) as suggested by the yellow color resulting from extensive colocalization with Texas Red-labeled lysosome-associated LAMP-1 (upper panels) and MIIC-associated HLA-DR (middle panels). However, as shown in the lower panels of Fig. 4, there was no colocalization with the major C-type lectin receptor CD206. Therefore it appears that, although the ECD/EGFR protein is highly glycosylated, internalization is not mediated through binding of carbohydrate moieties to this lectin receptor. In conclusion, the double immunofluorescence colocalization studies presented in Fig. 4 revealed efficient uptake and targeting to MHC class II processing compartments of the soluble protein antigen by the *in vitro* generated immature Mo-DC.

5.6. ECD/HER2-loaded Mo-DC exhibit strong immunostimulatory capacity

Since CD40-mediated signaling is important for appropriate T cell activation, surface expression of this key marker was evaluated on immature DC incubated for 3 days with either soluble ECD/HER2 or NIH3T3^{HER2} whole cell lysate. Flow cytometric analysis revealed differential expression of CD40 among these DC cultures. As presented in Fig. 5, almost all ECD/HER2-loaded DC expressed CD40 on the cell surface (panel A), in contrast to only about 50% of the NIH3T3^{HER2} lysate-loaded DC (panel B). Therefore, it was suggested that the former are more

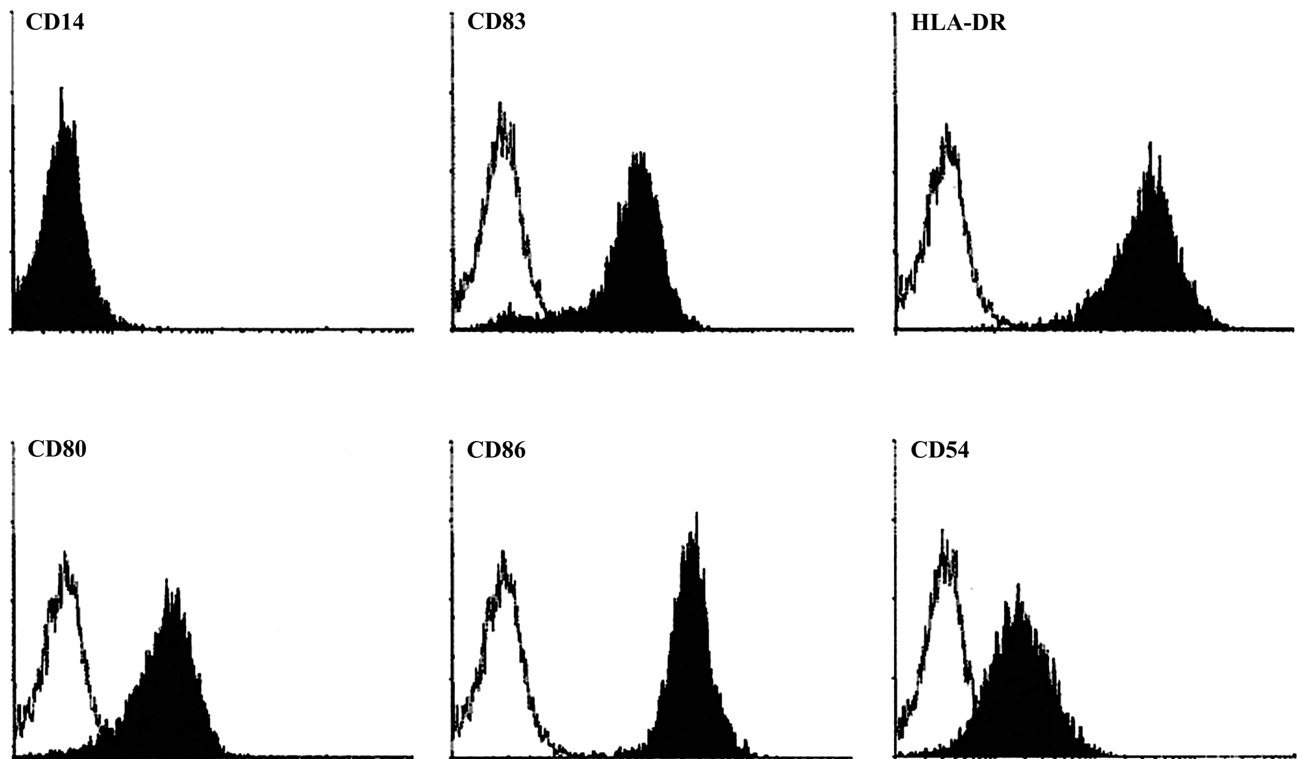

FIGURE 6: Phenotypic characterization of Mo-DC after maturation with proinflammatory cytokines and PGE_2. On day 10 of *in vitro* culture, matured Mo-DC were stained with mAb's specific for the indicated surface markers and analyzed by flow cytometry. Empty curves represent isotype-matched antibody controls.

potent in inducing antigen-specific T cell responses. This finding provided the rationale to use ECD/HER2-loaded DC as APC for the generation of antigen-specific T cells.

After maturation, ECD/HER2-pulsed Mo-DC were examined for surface expression of HLA-DR and other essential accessory molecules required for the induction and maintenance of T cell responses. As mentioned above and presented in Fig. 6, culture conditions caused a complete downregulation of CD14 and the induction of CD83. Furthermore, the high surface expression of the MHC-encoded HLA-DR molecule indicated the formation of large amounts of pMHC class II complexes. Besides pMHC-TCR interaction, a second signal delivered by the CD28-ligands CD80 and CD86 is required for the initiation of primary T cell responses. Since antigen-specific TCR-induction without costimulation leads to T cell anergy (Yi-qun Z. et al., 1997; Appleman L.J. and Boussiotis V.A., 2003), Mo-DC were analyzed for CD80 and CD86 and found to express elevated levels of these markers. In addition, CD54, an adhesion molecule that strongly interacts with LFA-1 on the T cell and drives development of primed $CD4^+$ T cells towards the Th1 phenotype (Smits H.H. et al., 2002), was highly abundant on the DC surface. Because Th1 cells support TAA-specific cytotoxic T cells to lyse tumor cells, this functional polarization is essential for the development of antitumor immunity. Finally, detection of a single peak for all tested markers in Fig. 6 demonstrated that treatment with proinflammatory cytokines and PGE_2 resulted in the homogeneous transformation of DC precursors to mature DC.

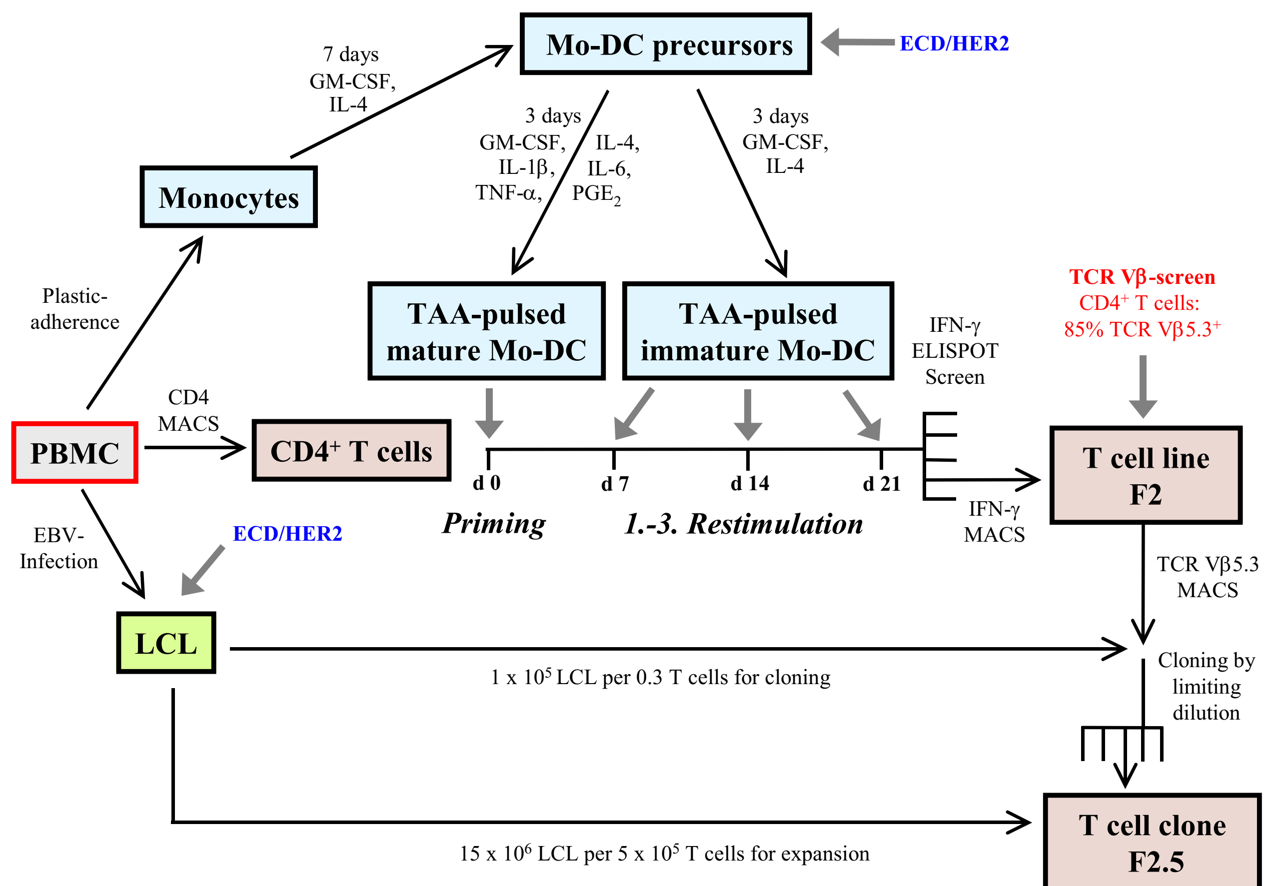

FIGURE 7: Illustration of the experimental procedure leading to the generation of the ECD/HER2-specific Th1 cell clone F2.5. DC were generated from blood monocytes, loaded with purified ECD/HER2 protein and used to stimulate MACS-isolated autologous CD4$^+$ T cells. After three cycles of weekly restimulations, T cell cultures were screened for ECD/HER2-reactivity by IFN-γ ELISPOT analysis. Microculture F2, displaying ECD/HER2 specificity, was selected for further cultivation. Flow cytometric analysis with a panel of TCR Vβ-specific mAb's revealed the usage of TCR Vβ5.3 by 85% of CD4$^+$ F2 T cells. TCR Vβ5.3$^+$ T cells were sorted by MACS and cloned by limiting dilution using ECD/HER2-loaded and γ-irradiated MaBa-LCL as stimulator cells. The CD4$^+$TCR Vβ5.3$^+$ T cell clone F2.5, demonstrating best viability and ECD/HER2-specific IFN-γ release, was expanded to large cell numbers by cultivation in T_{30}-flasks using ECD/HER2-loaded MaBa-LCL as APC.

5.7. Induction of primary responses represents a critical step in the isolation of HER2-specific T cells

A schematic overview of the procedure used to generate the ECD/HER2-specific CD4$^+$ T cell line F2 and the T cell clone F2.5 is given in Fig. 7. Mixed T cell-DC cultures were set up in 24-well plates with immunomagnetic bead-purified CD4$^+$ T cells and ECD/HER2-loaded autologous mature DC per well in a cell ratio of 100:1. Since the DC were highly motile and interact efficiently with T cells, as was observed by T cell-DC aggregate formation under the light microscope, the large excess of T cells appeared not to be detrimental for the initiation of antigen-specific primary responses. For a better detection of HER2 specificity in subsequent analyses, T cells in individual wells were maintained as separate cultures. Furthermore, resuspension was avoided for not to disrupt the colonies of proliferating T cells.

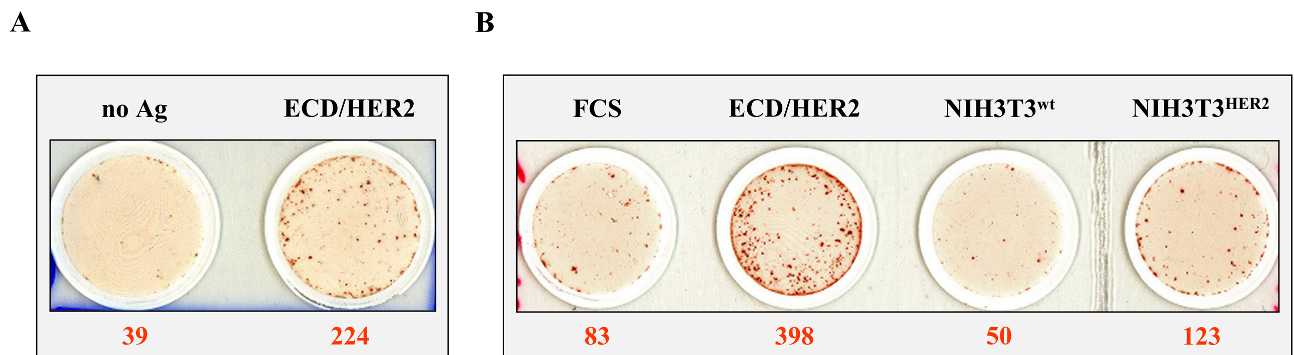

FIGURE 8: IFN-γ ELISPOT analysis of F2 T cells with antigen-pulsed immature Mo-DC as targets. For IFN-γ ELISPOT testing, 1 x 10^3 F2 T cells were cocultured with 2 x 10^4 antigen-loaded immature Mo-DC in microwells of a nitrocellulose filter plate precoated with the IFN-γ-specific mAb 1-D1K. After 20 h of incubation at 37°C in 5% CO_2 and removal of cells, captured IFN-γ was detected by the sequential addition of the biotinylated anti-IFN-γ mAb 7-B6-1, HRP-conjugated streptavidin and AEC substrate. Image analysis and spot enumeration with a gate set to exclude spots $<10^{-3}$ mm^2 were carried out using a dissecting microscope coupled with a computer-assisted video image analyzer. (**A**) Frequencies of IFN-γ secreting T cells directed against unloaded or ECD/HER2-loaded DC in the F2 T cell line 7 days after the second ECD/HER2-specific restimulation. (**B**) Data of a second immunospot analysis that was performed 7 days after the third restimulation with FCS, ECD/HER2, $NIH3T3^{wt}$ and $NIH3T3^{HER2}$ cell lysate as the antigens. Red numbers represent the calculated spot counts.

To prevent activation-induced death of the few antigen-specific T cells by overstimulation, all restimulations were performed with ECD/HER2-loaded immature instead of mature Mo-DC. The majority of T cells underwent apoptosis between the first and second restimulation. During this critical phase the T cells of the microcultures were transferred into U-bottom 96-well plates with the intention to create a protective microenvironment by direct contact of activated T cells. Another measure to protect TAA-specific T cells from cell death was the addition of the cytokine IL-7. As a result of this strategy, clusters of proliferating T cells surrounding the larger DC were observed under the light microscope in a few of the microcultures as soon as 3 days after the first restimulation.

5.8. The F2 T cell microculture depicts HER2-specific Th1 cell reactivity

Proliferating T cell cultures were tested after the second restimulation for HER2-specific IFN-γ secretion in an ELISPOT assay. Most of the cultures showed no differences in spot numbers between unpulsed and ECD/HER2-loaded immature DC, indicating that surface structures of the *in vitro* generated Mo-DC are more likely to activate T cells than it does the ECD/HER2 protein itself. Only a small fraction displayed more IFN-γ spots in the presence of ECD/HER2-pulsed DC. In particular, the T cell culture F2 exhibited high frequencies of activated T cells against ECD/HER2-loaded DC, but only low frequencies when the DC were unloaded. The image of this first ELISPOT analysis is presented in Fig. 8A. The same F2 T cell culture was analyzed after the

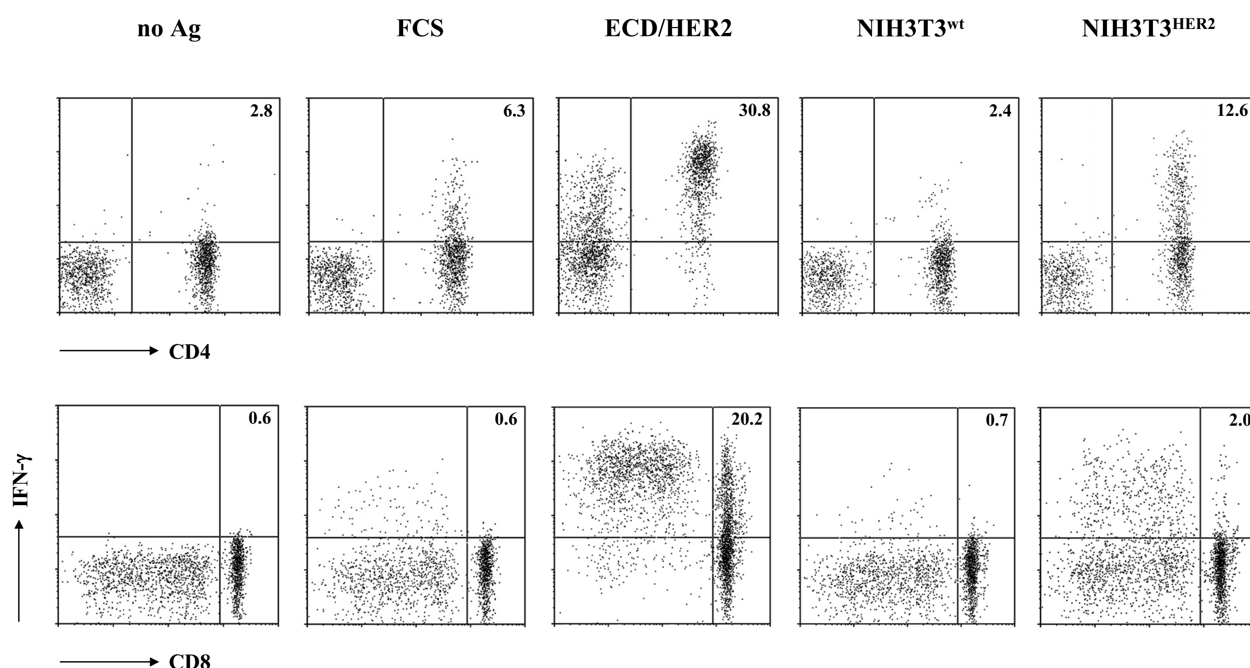

FIGURE 9: IFN-γ secretion of CD4$^+$ and CD8$^+$ T cells in the F2 T cell line upon antigen-presentation by immature Mo-DC. Following 18 h of coculture with antigen-loaded DC, F2 cells were subjected to IFN-γ secretion analysis and counterstained with anti-CD4 mAb 13B8.2 or anti-CD8 mAb B9.11. **Upper row**, CD4 staining of F2 T cells; **lower row**, CD8 staining of F2 T cells. The values in each box plot represent the relative number of IFN-γ secreting cells in percent.

third round of restimulation in a second ELISPOT assay using FCS and lysate of NIH3T3^{wt} cells as irrelevant and ECD/HER2 and lysate of NIH3T3^{HER2} cells as relevant antigens. As shown in Fig. 8B, the existence of HER2-reactive T cells in the F2 culture was confirmed by the detection of almost 5-fold more IFN-γ spots for ECD/HER2 than for FCS although the latter contains lots of potentially immunogenic proteins. Additional evidence was provided by the fact that the number of T cells displaying reactivity against NIH3T3 cell lysate was increased 2.5-fold by HER2 transfection.

IFN-γ secreting T cells in the F2 T cell line were isolated and maintained in culture by repeated stimulation with ECD/HER2-loaded DC. In order to improve the yield of F2 T cells, several cytokines and medium supplements were tested for their ability to enhance antigen-induced proliferation and to prevent apoptotic cell death. Medium containing 1% autologous plasma, 20 IU/ml IL-2, 5 ng/ml IL-7 and 5% T-Stim, a conditioned medium from activated human PBMC, was judged to best fulfill these requirements.

As soon as sufficient cell numbers were reached under the optimized culture conditions, F2 T cells were further analyzed for antigen-specificity using a matrix-based IFN-γ secretion assay. This flow cytometric assay allowed a more thorough characterization of activated T cells compared to the ELISPOT technique by additional analysis of cell surface marker expression. As displayed in Fig. 9, the measurements revealed that the F2 T cell line consisted of two distinct T cell populations since 40% of the cells were bright CD4$^+$ (upper panel) and 60% bright CD8$^+$ (lower panel). However, the most striking observation of this analysis was a diffuse expression of CD8

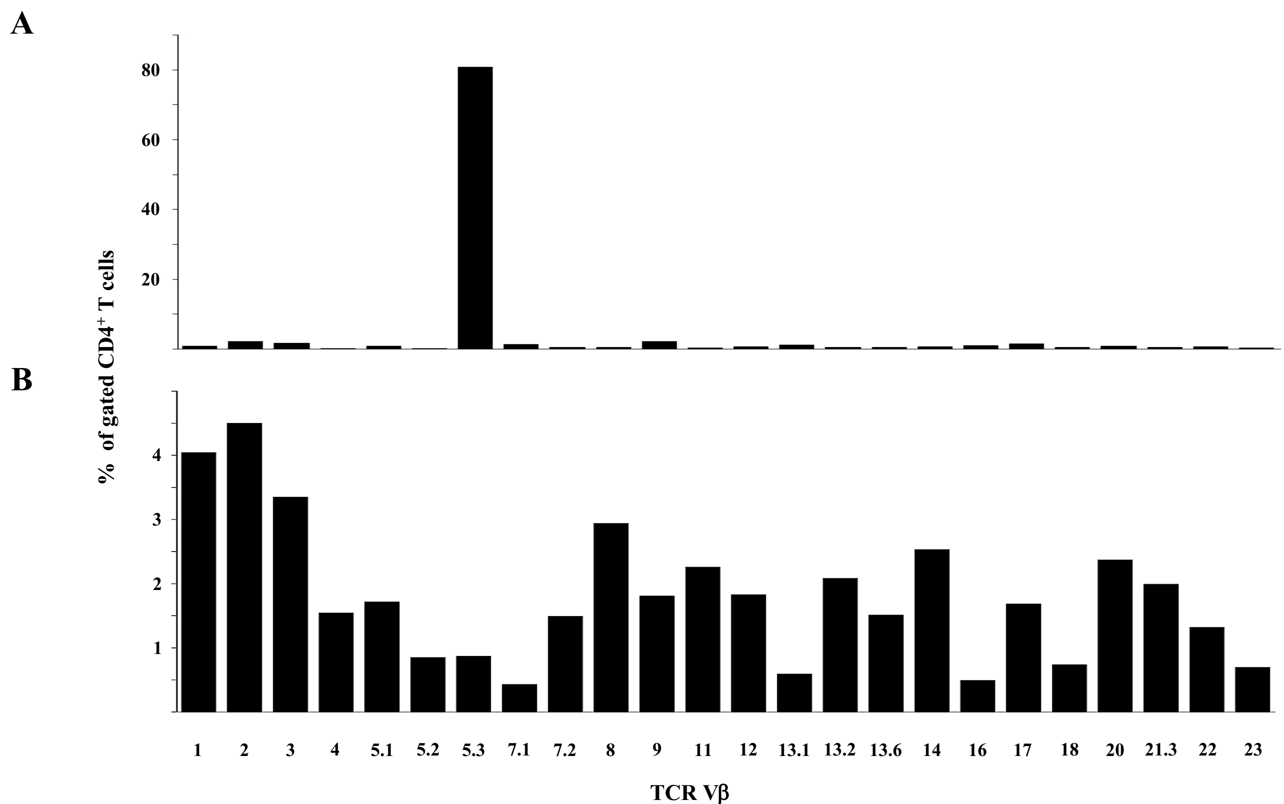

FIGURE 10: TCR Vβ expression pattern of CD4^{+} T cells in the F2 T cell line and in PBMC from donor MaBa. Viable (PI^{-}) cells were gated on CD4 bright and analyzed with a panel of TCR Vβ-specific mAb's covering about 60% of the TCR Vβ repertoire of T lymphocytes from human subjects. Clonogram of (**A**) CD4^{+}CD8^{lo} F2 T cells and (**B**) CD4^{+} bright gated cells in uncultured *ex vivo* PBMC from donor MaBa.

also by the CD4^{+} T cells. According to this, the two subsets were designated as CD4^{+}CD8^{lo} and CD4^{-}CD8^{+} F2 T cells. As shown in Fig. 9, ECD/HER2 presentation by DC induced IFN-γ release by both the CD4^{+}CD8^{lo} and the CD4^{-}CD8^{+} T cell population, but with much higher levels for the former. The same was observed, although to a much lesser extent, for whole protein extract of NIH3T3^{HER2} cells. FCS as a control induced only modest IFN-γ release by CD4^{+}CD8^{lo} T cells and had no effect on CD4^{-}CD8^{+} T cells. Neither CD4^{+}CD8^{lo} nor CD4^{-}CD8^{+} F2 T cells produced IFN-γ in culture with unloaded DC or DC loaded with NIH3T3^{wt} cell lysate. Thus, the IFN-γ secretion analysis presented in Fig. 9 suggested HER2 specificity of both the CD4^{+}CD8^{lo} and the CD4^{-}CD8^{+} F2 T cell subset.

5.9. HER2-specific CD4^{+}CD8^{lo} F2 T cells are TCR Vβ5.3^{+} and exhibit effector phenotype

CD4^{+} positively selected T cells from a PBMC preparation were used as starting material for the induction of a HER2-specific immune response. Thus, it became obvious that the CD4^{-}CD8^{+} T cells although ECD/HER2-specific represent a contamination either of the initial CD4^{+} T cell isolate or of one of the monocyte preparations used to generate ECD/HER2-loaded DC. Based on

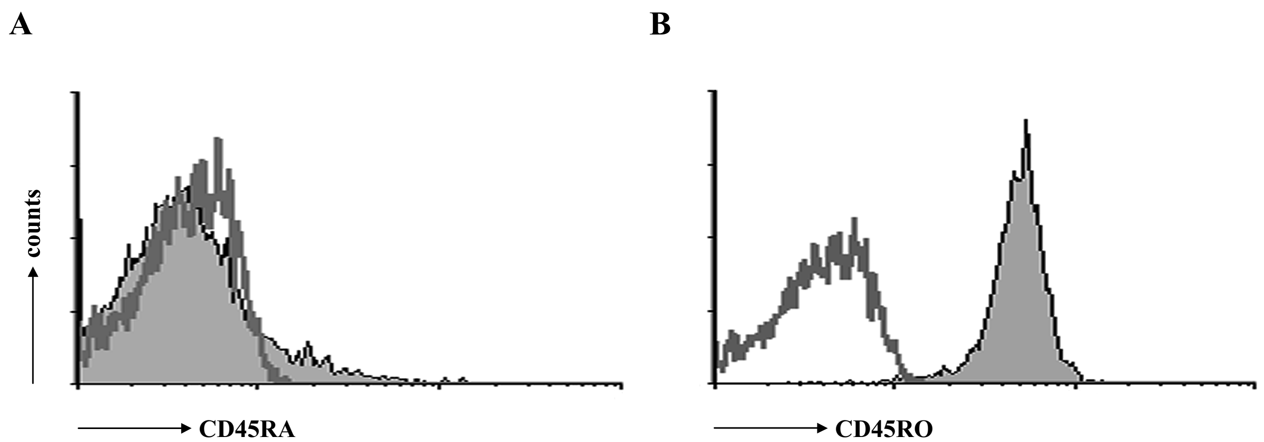

FIGURE 11: CD45RO and CD45RA expression on TCR Vβ5.3^{+} F2 T cells. TCR Vβ5.3^{+} F2 T cells were immunostained with either the CD45RA-specific mAb HI100 or the CD45RO-specific mAb UCHL1 and analyzed by flow cytometry. Histograms for (**A**) CD45RA expression and (**B**) CD45RO expression. Empty curves represent the isotype-matched antibody control.

the assumption that also in the future T, B or NK cells might be introduced in the T cell culture and sooner or later overgrow HER2-specific T cells it was of particular interest to find a reliable method for the unambiguous identification of the HER2-specific F2 Th cells at this critical stage of T cell cultivation. One possibility could be the characterization of the TCR used by the HER2-specific T cells. For this purpose, a flow cytometric analysis with a panel of TCR Vβ-specific antibodies was established that permitted the determination of 24 TCR Vβ specificities representing almost 60% of T cell frequencies in the blood of normal individuals. Whereas no predominant TCR Vβ motif was evident on CD4^{-}CD8^{+} F2 T cells, analysis of CD4^{+}CD8^{lo} F2 T cells clearly showed that 85% of them were positive for TCR Vβ5.3 (Fig. 10A). Besides this high TCR Vβ5.3 chain frequency, the distribution of all other common TCR Vβ types was less than 2%. For comparison, Fig. 10B depicts the TCR Vβ usage of CD4^{+} T cells in PBMC from the original

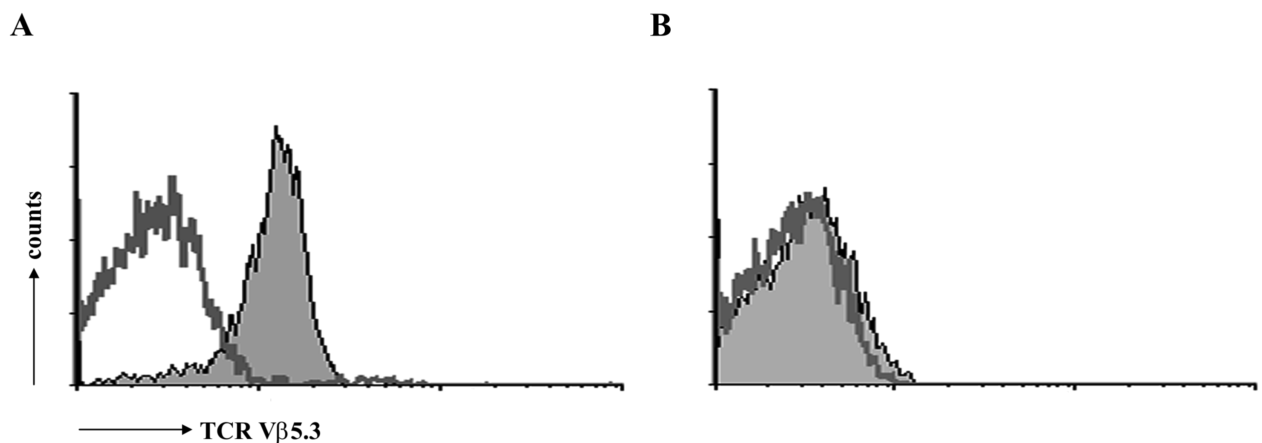

FIGURE 12: Restriction of TCR Vβ5.3 expression to CD4^{+} T cells in the F2 T cell line. Lymphocytes were gated on CD4^{bright} or CD8^{bright} and analyzed for TCR Vβ5.3 surface expression using the PE-labeled mAb 3D11. Histograms for (**A**) CD4^{bright} F2 T cells and (**B**) CD8^{bright} F2 T cells. Empty curves represent the isotype-matched antibody control.

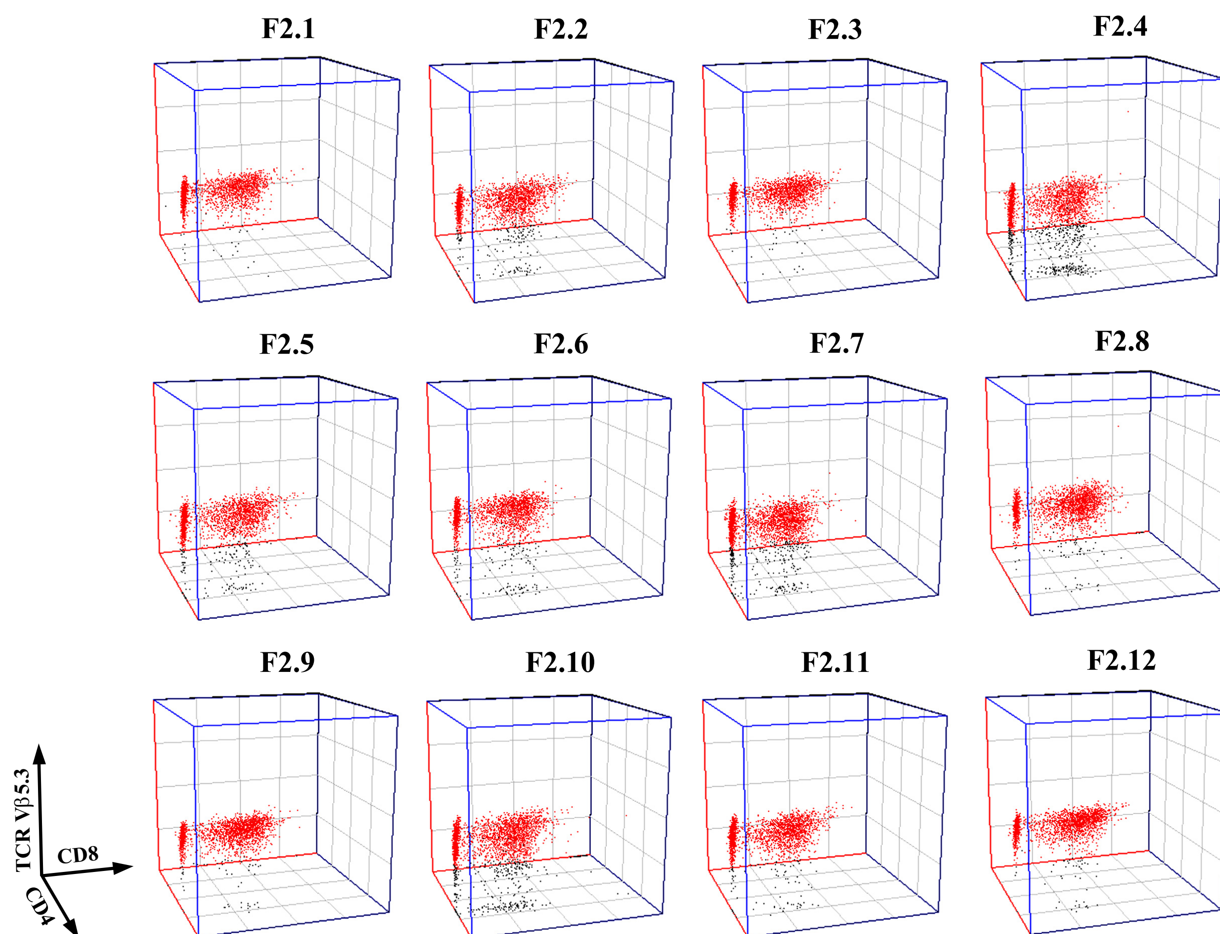

FIGURE 13. Expression of CD4, CD8 and TCR Vβ5.3 on F2 T cell line-derived T cell clones. TCR Vβ5.3$^+$ F2 cells were isolated by MACS and cloned by limiting dilution in 96-well plates using 0.3 T cells and 1 x 10^5 ECD/HER2-pulsed γ-irradiated MaBa-LCL feeder cells per well. After 2 weeks of culture, the resulting 12 clones were analyzed for CD4, CD8 and TCR Vβ5.3 expression. Data for each clone is given as three-color dot plot. TCR Vβ5.3 positivity is shown in red.

donor MaBa. The 80-fold increase in TCR Vβ5.3$^+$ T cell numbers was taken as a proof that one single T cell in the F2 microculture had undergone clonal selection and expansion.

The next question concerned the activation status of TCR Vβ5.3$^+$ F2 T cells. Since alternative splicing of the membrane protein phosphatase CD45 is regulated in such a way that naive T cells predominantly express the CD45RA isoform and switch to CD45RO after activation (Akbar A.N. et al., 1988), CD45 isoform expression was used as an indicator. As shown in Fig. 11, TCR Vβ5.3$^+$ F2 T cells were completely negative for CD45RA (panel A) and positive for CD45RO (panel B), and therefore appeared to be activated memory T cells.

Clonal selection and differentiation into an effector phenotype clearly supports ECD/HER2-specificity of CD4$^+$TCR Vβ5.3$^+$ T cells in the F2 T cell culture. Therefore, a flow cytometric analysis on both the CD4$^+$CD8^{lo} and CD4$^-$CD8$^+$ F2 T cell subset was initiated to prove the feasibility of a TCR Vβ5.3-based isolation strategy. As presented in Fig. 12, the obtained results revealed that the TCR Vβ5.3 motif is exclusively expressed on CD4$^+$ F2 T cells. Therefore, TCR Vβ5.3$^+$ F2 T cells were sorted and cloned by limiting dilution.

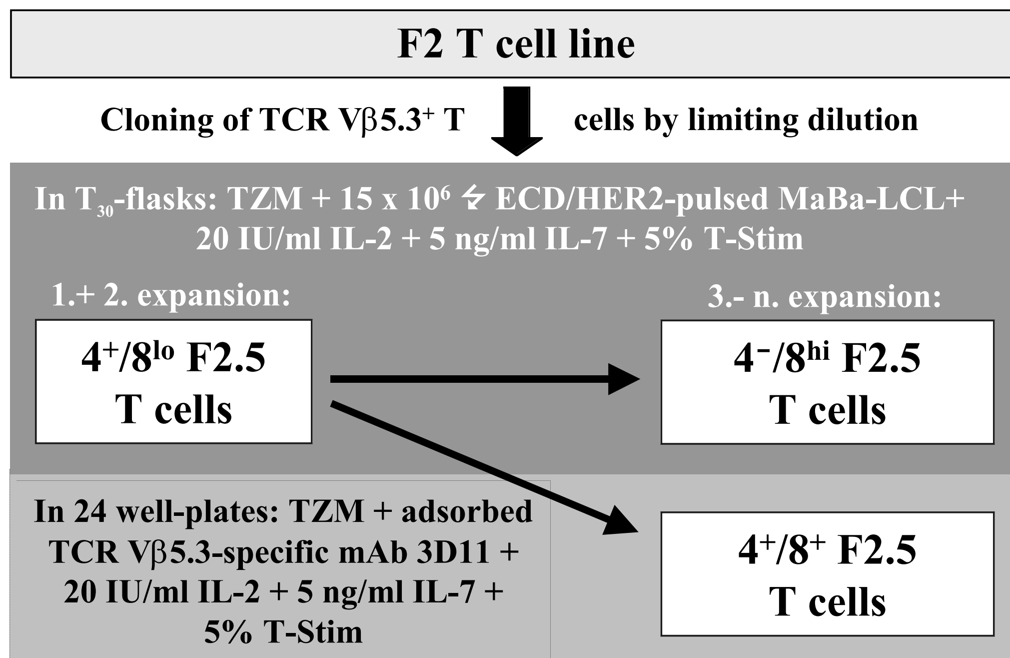

FIGURE 14: Overview of the established culture conditions for *in vitro* expansion of the F2.5 T cell clone. After cloning and a first expansion in T_{30}-flasks with ECD/HER2-pulsed γ-irradiated MaBa-LCL, T cells were characterized by $CD4^{bright}$ and $CD8^{dim}$ expression. Due to this, cells were named $4^+/8^{lo}$ F2.5 T cells. After two further rounds of expansion, T cells lost detectable expression of CD4 and stably upregulated CD8. For this reason, cells were designated as $4^-/8^{hi}$ F2.5 T cells. $4^+/8^{lo}$ F2.5 T cells were alternatively expanded in the wells of 24-well plates precoated with the TCR Vβ5.3-specific agonistic mAb 3D11. By use of this method, T cells developed a phenotype characterized by simultaneous surface expression of CD4 and CD8 and were therefore named $4^+/8^+$ F2.5 T cells.

5.10. The Th cell clone F2.5 displays altered coreceptor expression dependent on culture conditions

Twelve T cell clones were obtained by limiting dilution cloning of TCR Vβ5.3-sorted F2 T cells. As suggested by the three-color dot plots in Fig. 13 all T cell clones had the same expression profile for CD4, CD8 and TCR Vβ5.3. In the subsequent cultivation procedure, 11 of these clonal microcultures could not be further expanded and all T cells progressively died within two weeks. Only one clone, designated as F2.5, depicted good viability and long-term proliferation capacity.

By incubation with ECD/HER2-pulsed autologous LCL (designated as MaBa-LCL), F2.5 T cells could be expanded 20-fold during a two week culture period. Because the antigen-loaded MaBa-LCL were γ-irradiated, the F2.5 T cells were the only proliferating cells in the culture. F2.5 T cells were characterized by flow cytometric analysis and, like in the parental F2 T cell line, found to express high levels of CD4 and moderate levels of CD8. Therefore, these cells were named $4^+/8^{lo}$ F2.5 T cells. Strikingly, after two further rounds of expansion in suspension culture, cells lost CD4 and expressed CD8 at high levels. After the phenotypic switch, these $4^-/8^{hi}$ F2.5 T cells could be maintained stably in culture. A portion of the $4^+/8^{lo}$ F2.5 T cells was alternatively cultivated in 24-well plates precoated with the TCR Vβ5.3-specific agonistic mAb 3D11. As a

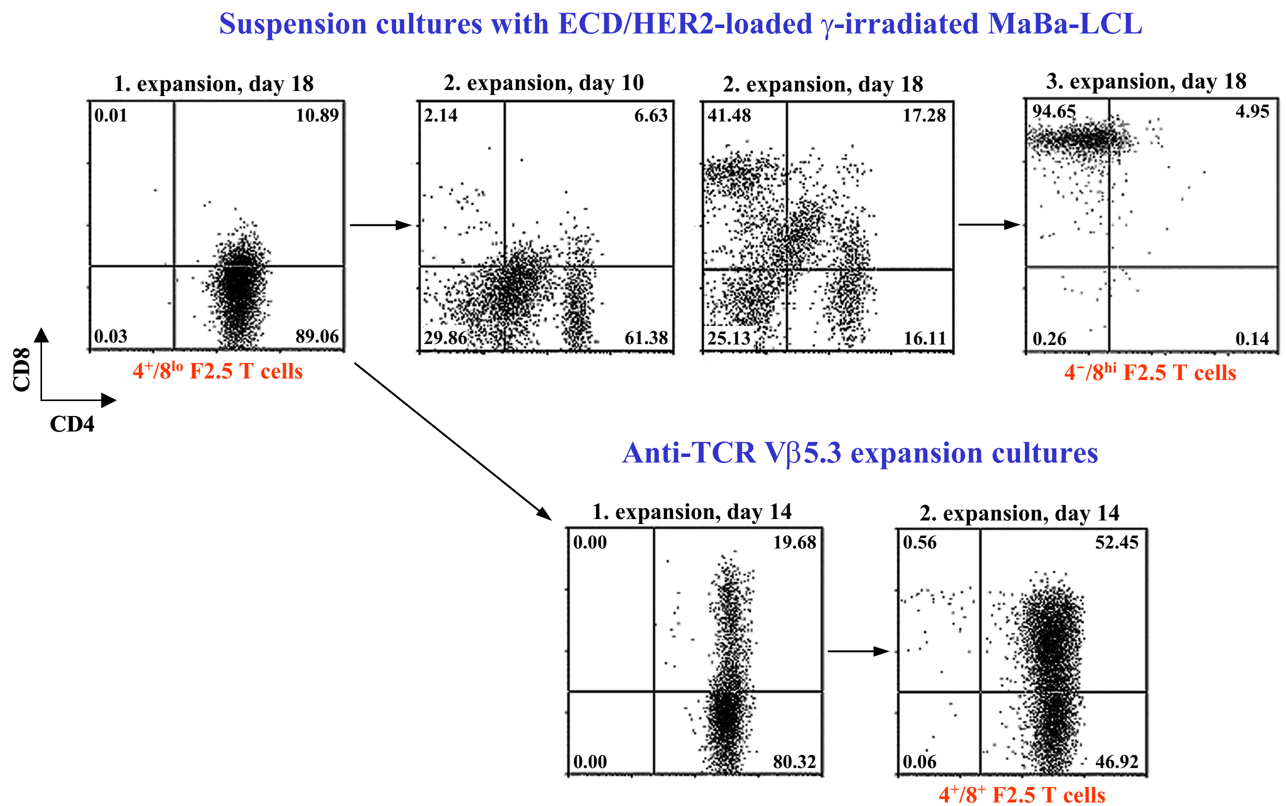

FIGURE 15: Analysis of CD4 and CD8 surface expression during the early stage of F2.5 T cell clone culture. F2.5 T cells of the LCL- and the anti-TCR Vβ5.3-stimulated cultures were harvested at the indicated time points and immunostained with anti-CD4 mAb 13B8.2 and anti-CD8 mAb B9.11 for flow cytometric analysis. In the two-color dot plots, expression of CD4 is given on the x-axis and CD8 on the y-axis. The values represent the relative numbers of cells in each quadrant.

result of this antibody-induced T cell activation, cells developed a phenotype characterized by simultaneous expression of both CD4 and CD8 and were therefore designated $4^+/8^+$ F2.5 T cells. A schematic overview of the cell culture procedures for F2.5 T cell clone expansion and their effects on the T cells phenotype is given in Fig. 14.

Based on the fact that coculture of $4^+/8^{lo}$ F2.5 T cells with ECD/HER2-loaded MaBa-LCL consistently led to a $CD8^+$ single positive (SP) phenotype, the course of F2.5 T cell clone transition was revealed by flow cytometric detection of CD4 and CD8 expression levels at different times of expansion culture. As presented in Fig. 15, F2.5 T cells maintained a $4^+/8^{lo}$ F2.5 phenotype in a first round of T_{30}-flask expansion. On day 10 of the second expansion, CD4 was markedly downregulated on most of the cells, whereas only 2% of the F2.5 T cells expressed CD8. However, 8 days later the $CD8^+$ fraction was significantly increased in numbers. At this stage, two other populations were detected in the culture, the one displaying a $CD4^-CD8^-$ double negative (DN) phenotype and the other a $CD4^{lo}CD8^{lo}$ phenotype. Whereas it seemed that the latter is an intermediate on the way to the stable $4^-/8^{hi}$ F2.5 phenotype, it is not clear if DN cells also acquired this phenotype or died by apoptosis. Conversely, when $4^+/8^{lo}$ F2.5 T cells were expanded in anti-TCR Vβ5.3-coated cell culture plates, CD4 levels remained unchanged, whereas CD8 levels became upregulated and remained stable throughout the whole culture period.

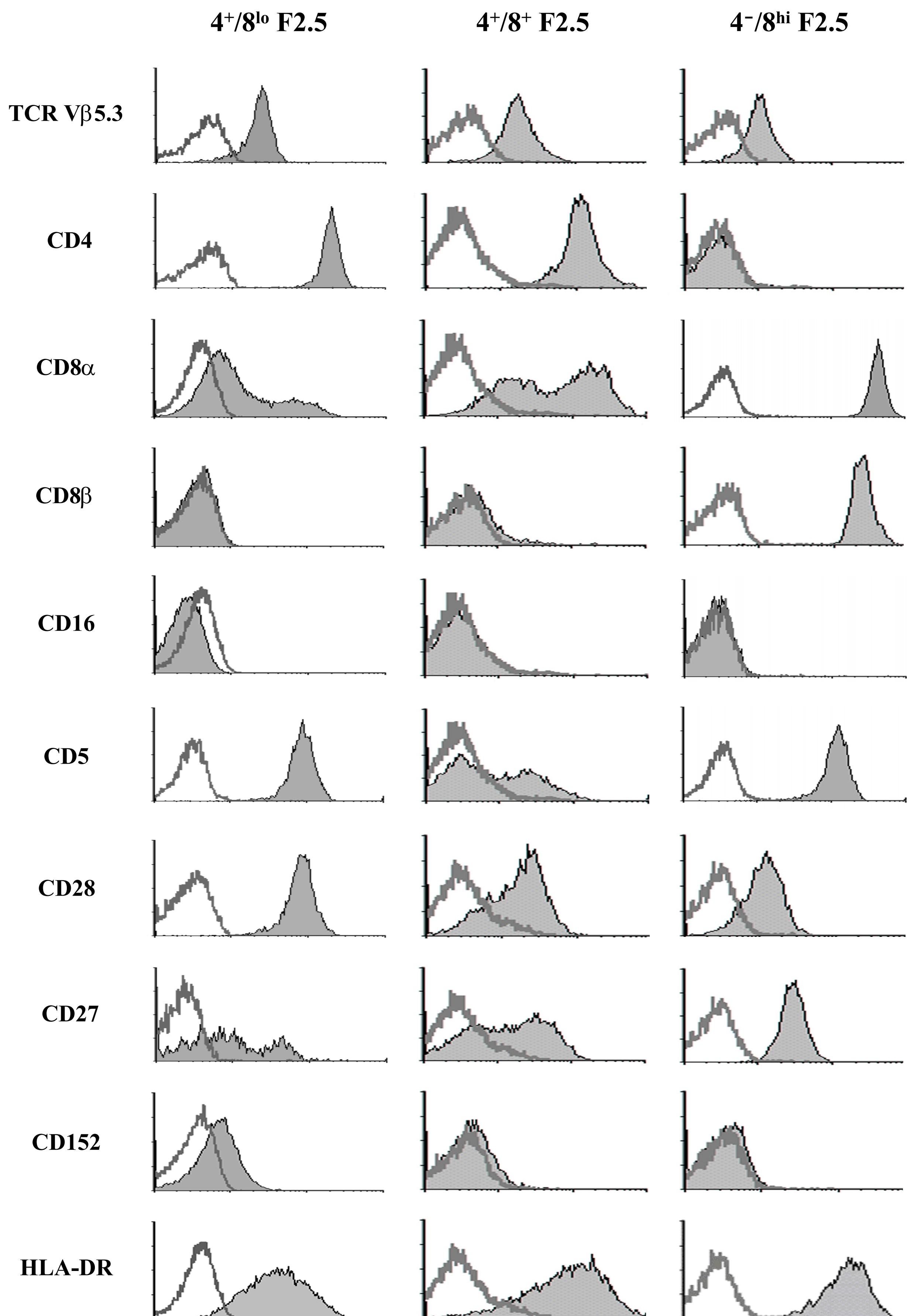

FIGURE 16: Flow cytometric analysis of surface marker expression on the F2.5 T cell clone isoforms. A total of 1 x 10^5 stained cells were included in each analysis. The shaded histogram represents staining with a mAb specific for the surface antigen indicated on the left margin, whereas the open histogram depicts staining with an isotype-matched mAb of irrelevant specificity. On the x-axis the fluorescence intensity and on the y-axis the relative cell number is indicated.

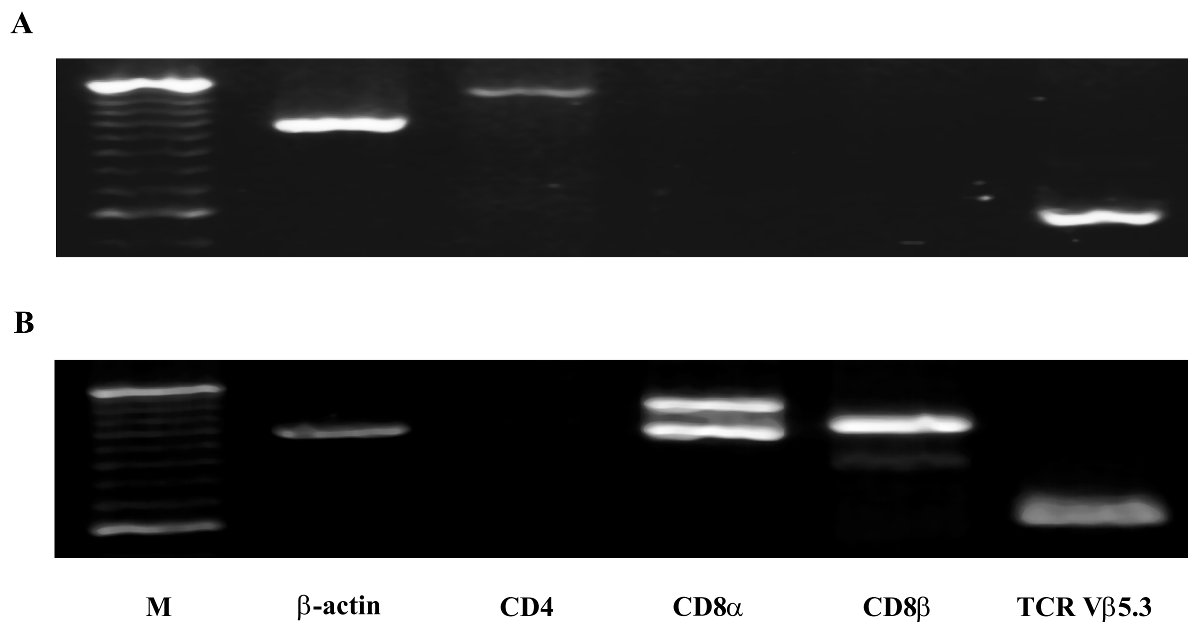

FIGURE 17: RT-PCR analysis of CD4, CD8α, CD8β and TCR Vβ5.3 mRNA expression in $4^+/8^{lo}$ and $4^-/8^{hi}$ F2.5 T cells. RT-PCR was performed as described in material and methods using total cellular RNA of 1 x 10^6 T cells and the specific primer pairs presented in table 1. PCR products were resolved in a 2% agarose gel and analyzed for ethidium bromide staining. Agarose gel image for (**A**) $4^+/8^{lo}$ F2.5 T cells and (**B**) $4^-/8^{hi}$ F2.5 T cells. **M**, 50 bp-ladder DNA marker.

$4^-/8^{hi}$ F2.5 T cells died within a few days, when they were transferred from the T_{30}-flasks to anti-TCR Vβ5.3-coated culture plates. It was, however, possible at any time to convert anti-TCR Vβ5.3 expanded $4^+/8^+$ F2.5 T cells to $4^-/8^{hi}$ F2.5 T cells by transferal to suspension culture.

5.11. The F2.5 T cell clone isoforms reveal substantial phenotypic differences

A detailed immunophenotypic analysis of the F2.5 T cell clone isoforms $4^+/8^{lo}$, $4^+/8^+$ and $4^-/8^{hi}$ is presented in Fig. 16. Overall expression of the TCR Vβ5.3 chain was taken as a sign that all three T cell subsets were derived from the same precursor cell. For retrieval of previous data, CD4 was found in high levels on the surface of $4^+/8^{lo}$ and $4^+/8^+$ F2.5 T cells, but was completely absent on $4^-/8^{hi}$ F2.5 T cells. Staining for the α-chain of the CD8 coreceptor revealed two distinct subsets of T cells with different expression levels in the $4^+/8^{lo}$ and $4^+/8^+$ F2.5 T cell culture, whereas $4^-/8^{hi}$ F2.5 T cells were characterized by uniformly high CD8α levels. Expression of the CD8β chain was only detected on $4^-/8^{hi}$ F2.5 T cells. To rule out the possibility that F2.5 cells might be natural or IL-2-activated killer cells (Trinchieri G., 1989), CD16 expression was analyzed with the finding that all three T cell isoforms were negative for this marker. Detection of CD5, a common inhibitory receptor on T and B cells (Ravetch J.V. and Lanier L.L., 2000), demonstrated strong expression on $4^+/8^{lo}$ and $4^-/8^{hi}$, but only weak expression on $4^+/8^+$ F2.5 T cells. An approximately 10-fold reduction in CD28 expression levels occurred during the course of $4^+/8^{lo}$ to $4^-/8^{hi}$ F2.5 T cell transition, although CD28 was present in high density on all three

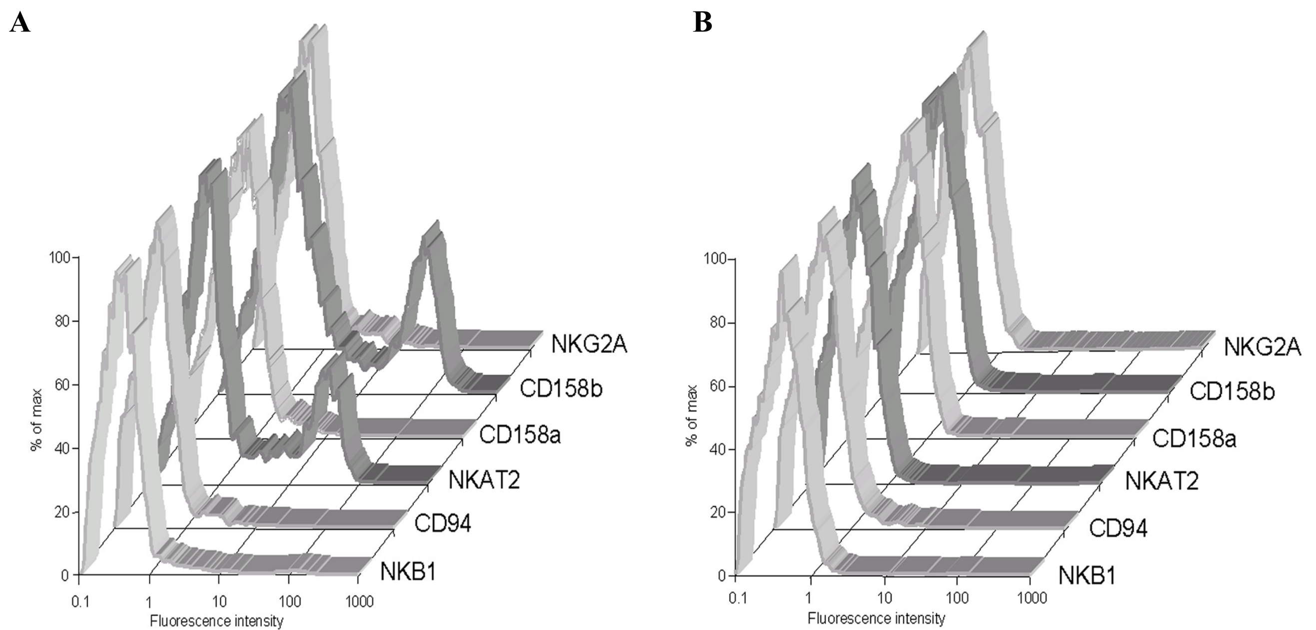

FIGURE 18: KIR expression on 4⁻/8^{hi} and 4⁺/8⁺ F2.5 T cells. Antibodies used for the flow cytometric analysis were anti-NKB1 (DX9), anti-CD94 (HP-3D9), anti-NKAT2 (DX27), anti-CD158a (EB6), anti-CD158b (CH-L) and anti-NKG2A (Z199). Histogram presentation for (**A**) 4^-/8^{hi} F2.5 T cells and (**B**) $4^+/8^+$ F2.5 T cells. **KIR synonyms:** NKB1 = 3DL1, CD158a = 2DL1 and 2DS1, NKAT2 = CD158b = 2DL2, 2DL3 and 2DS2; CD94 and NKG2A, subunits of the hetero-dimeric C-type lectin receptor.

T cell isoforms. Interestingly, the decrease in CD28 levels was accompanied by an increase in CD27 surface expression. Since CD152 competes with CD28 for the binding of CD80 and CD86 on APC, thereby preventing T cell activation (Reiser H. and Stadecker M.J., 1996; Sayegh M.H. and Turka L.A.,1998), this marker was also included in the analysis and was found to be weakly expressed on $4^+/8^{lo}$ F2.5 T cells and absent on the other two isoforms. Finally, the detection of high HLA-DR surface levels revealed an activated phenotype of all three T cell clone isoforms.

5.12. $4^-/8^{hi}$ F2.5 T cells selectively express KIR 2DL2 and soluble CD8α

Flow cytometric data revealed the transition of the HER2-specific T cell clone F2.5 from a Th to a Tc phenotype. Since these results are contrary to the usual assumption that only mature T cells leave the thymus and circulate in the periphery, coreceptor expression of $4^+/8^{lo}$ and $4^-/8^{hi}$ F2.5 T cells was verified by RT-PCR. As a specificity control, TCR Vβ5.3 mRNA detection was also included in these analyses. As presented in Fig. 17, $4^+/8^{lo}$ F2.5 T cells were positive for CD4 and TCR Vβ5.3 mRNA (panel A), whereas $4^-/8^{hi}$ F2.5 T cells contained CD8α, CD8β and TCR Vβ5.3 mRNA (panel B). Remarkably, two CD8α transcripts of different length were amplified by the specific primer pair indicating alternative splicing and as a consequence expression of both a membrane-bound and a soluble form of the CD8α molecule (Giblin P. et al., 1989).

Another important observation was KIR surface expression by some of the $4^-/8^{hi}$ F2.5 T cells.

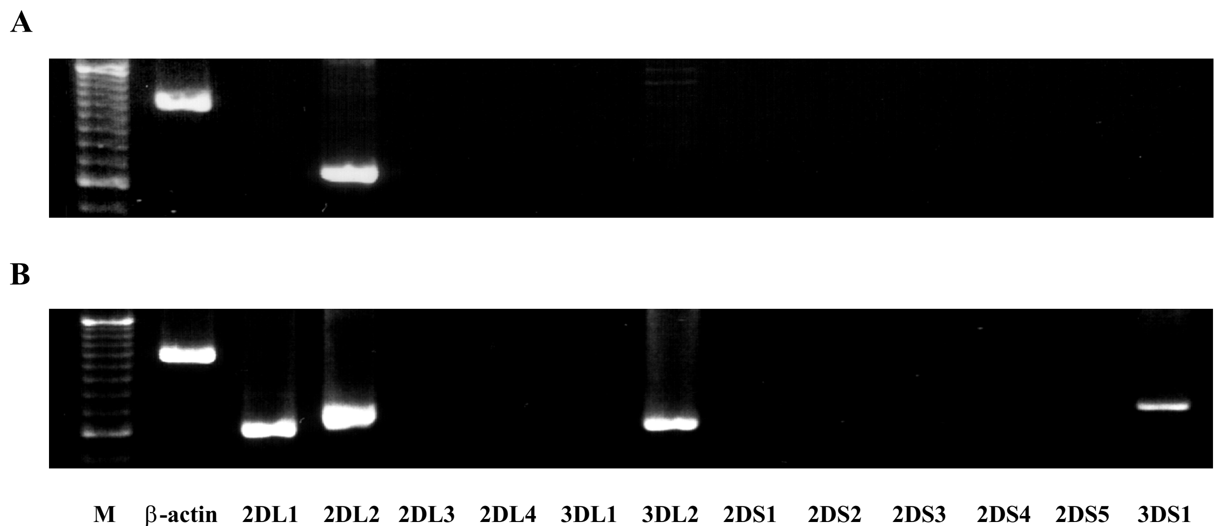

FIGURE 19: KIR transcripition pattern of $4^-/8^{hi}$ F2.5 T cells and PBMC of donor MaBa. The oligonucleotide primers used for RT-PCR analysis were designed according to the conserved KIR sequences given by Uhrberg M. et al. (1997) and are presented in table 1. PCR samples were resolved in a 2% agarose gel and analyzed for ethidium bromide staining. Agarose gel image for (**A**) $4^-/8^{hi}$ F2.5 T cells and (**B**) PBMC of donor MaBa. **M**, 50 bp-ladder DNA marker.

The first evidence was provided by flow cytometric analysis with a panel of KIR specific mAb's. As presented in Fig. 18, 20% of $4^-/8^{hi}$ F2.5 T cells showed intense staining for NKAT2 and CD158b (panel A), whereas none of the analyzed KIR was detectable on $4^+/8^+$ F2.5 T cells (panel B). Since NKAT2 and CD158b designate a family of KIR, RT-PCR analysis was consecutively performed for a more accurate KIR detection. As shown in Fig. 19, RT-PCR analysis identified exclusively KIR 2DL2 mRNA expression in $4^-/8^{hi}$ F2.5 T cells (panel A). The complete KIR repertoire of donor MaBa was determined in parallel using total RNA from freshly isolated PBMC as the template. RT-PCR analysis of PBMC revealed the expression of KIR 2DL1, 2DL2, 3DL2 and 3DS1 (panel B).

5.13. All F2.5 T cell clone isoforms produce IFN-γ after stimulation with ECD/HER2-loaded Mo-DC

The T cell clone isoforms were analyzed for ECD/HER2-specific IFN-γ secretion by flow cytometry. In addition to IFN-γ, cells were stained for CD4 and CD8 surface expression. Fluorescence data were displayed as three-color dot plots with CD8 on the x-, IFN-γ on the y- and CD4 on the z-axis. Data acquisition and evaluation was performed for comparison under the same conditions and settings for all three isoforms.

As presented in Fig. 20, $4^+/8^{lo}$ F2.5 T cells responded well to ECD/HER2 protein expressed in either CHO or L cells. However, the broad distribution of fluorescence along the y-axis indicates that the extent of IFN-γ release in response to ECD/HER2 varied markedly among the cells. In contrast, KLH as an irrelevant protein antigen, FCS representing a mixture of many xenogenic proteins, and ECD/EGFR as an ECD/HER2-related protein, expressed and purified identically, induced only weak IFN-γ responses. In the case of $4^+/8^+$ F2.5 T cells, presentation of CHO cell-

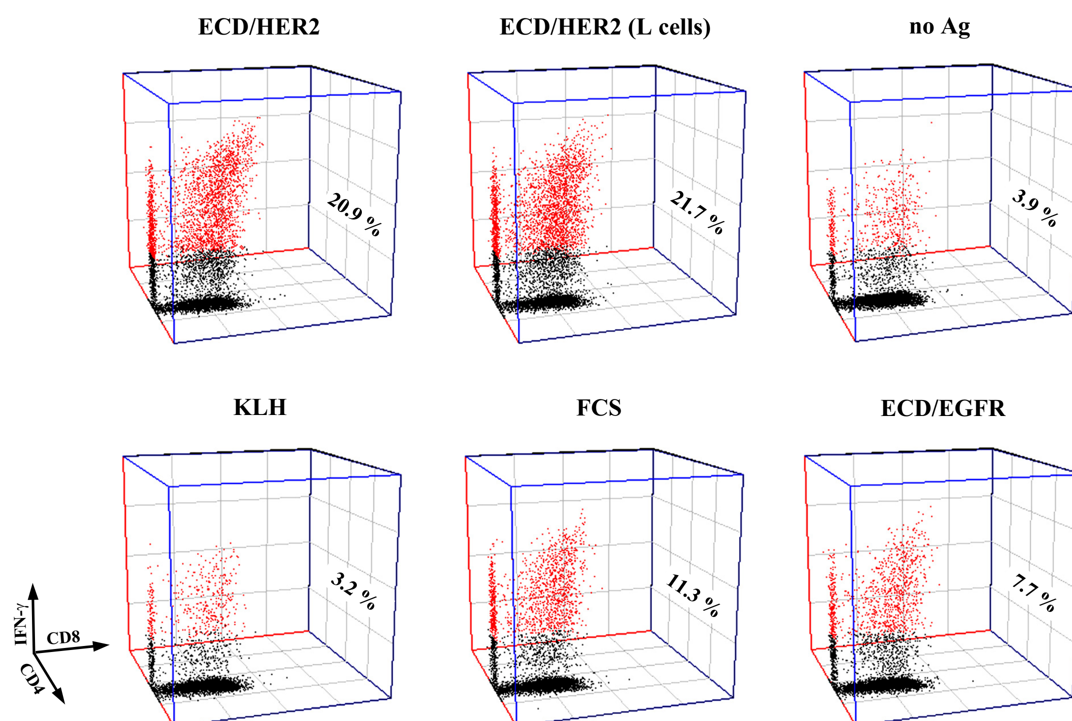

FIGURE 20: IFN-γ secretion profile of the $4^{+}/8^{lo}$ F2.5 T cell clone isoform upon antigen-presentation by immature Mo-DC. Following 18 h of coculture with antigen-loaded Mo-DC, $4^{+}/8^{lo}$ F2.5 T cells were subjected to IFN-γ secretion analysis, as described in material and methods, and counterstained with the CD4-specific mAb 13B8.2 and the CD8-specific mAb B9.11. In the three-color dot plots, expression of CD8 is given on the x-axis, IFN-γ on the y-axis and CD4 on the z-axis. The percentages of IFN-γ secreting cells (red) were estimated on the basis of a discriminating gate. Values are displayed in the plots.

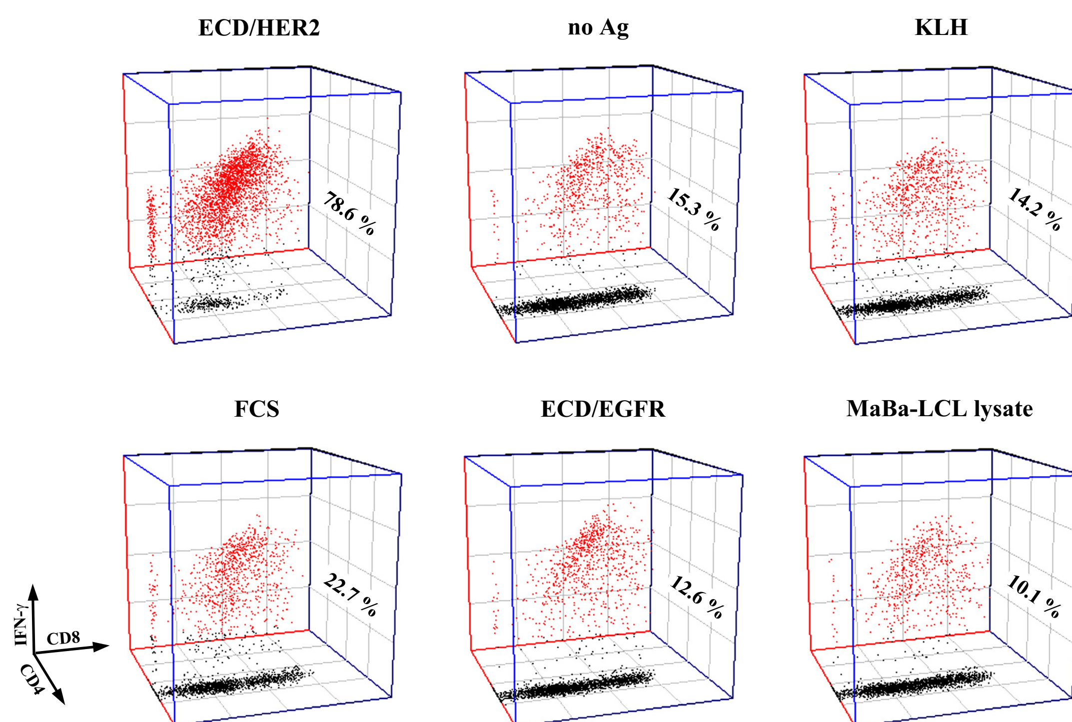

FIGURE 21: IFN-γ secretion profile of the $4^{+}/8^{+}$ F2.5 T cell clone isoform upon antigen-presentation by immature Mo-DC. Experimental procedure and data evaluation was the same as described for the $4^{+}/8^{lo}$ F2.5 T cell clone isoform in figure 20.

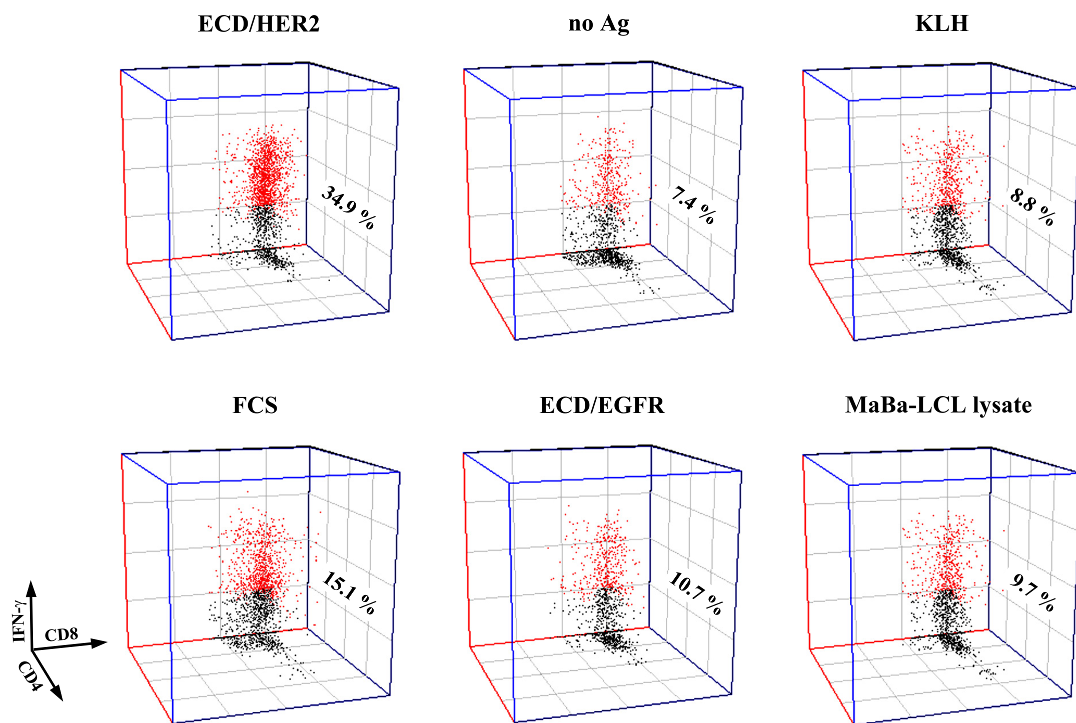

FIGURE 22: IFN-γ secretion profile of the $4^-/8^{hi}$ F2.5 T cell clone isoform upon antigen-presentation by immature Mo-DC. Experimental procedure and data evaluation was the same as described for the $4^+/8^{lo}$ F2.5 T cell clone isoform in figure 20.

derived ECD/HER2 antigen by immature Mo-DC led to virtually complete activation with high IFN-γ expression levels (Fig. 21). However, responsiveness of $4^+/8^+$ F2.5 T cells was generally increased, since the appropriate dot plots in Fig. 21 reveal elevated numbers of IFN-γ secreting T cells also in the cultures with unloaded DC or DC pulsed with the irrelevant antigens KLH, FCS, ECD/EGFR and MaBa-LCL lysate. As shown in Fig. 22, an identical profile of IFN-γ responses was observed for $4^-/8^{hi}$ F2.5 T cells with numbers of activated cells similar to those of $4^+/8^{lo}$ F2.5 T cell cultures. From these flow cytometric analyses, it may be deduced that ECD/HER2 recognition by the F2.5 T cell clone was not abrogated by the extensive phenotypic changes that occur during T cell culture.

The F2.5 T cell clone isoforms were additionally tested for their capacity to discriminate between NIH3T3^{HER2} and NIH3T3^{wt} cells. Since TAA-specific T cell activation by DC that had phagocytosed apoptotic tumor cells may represent more closely the *in vivo* situation at the tumor site, this approach is more appropriate for the assessment of antigen sensitivity than the one with purified protein antigens. As illustrated in Fig. 23, NIH3T3^{HER2} cell lysate (upper dot plots) led to a 2-fold increase in the numbers of IFN-γ secreting $4^+/8^{lo}$ and $4^+/8^+$ F2.5 T cells when compared to NIH3T3^{wt} cell lysate (lower dot plots). Again, the responsiveness to both cell lysate preparations was markedly higher for the $4^+/8^+$ F2.5 T cell clone isoform. A completely different pattern was found for $4^-/8^{hi}$ F2.5 T cells. Whereas these cells became activated in response to ECD/HER2, lysate of NIH3T3^{HER2} cells failed to induce a HER2-specific IFN-γ response.

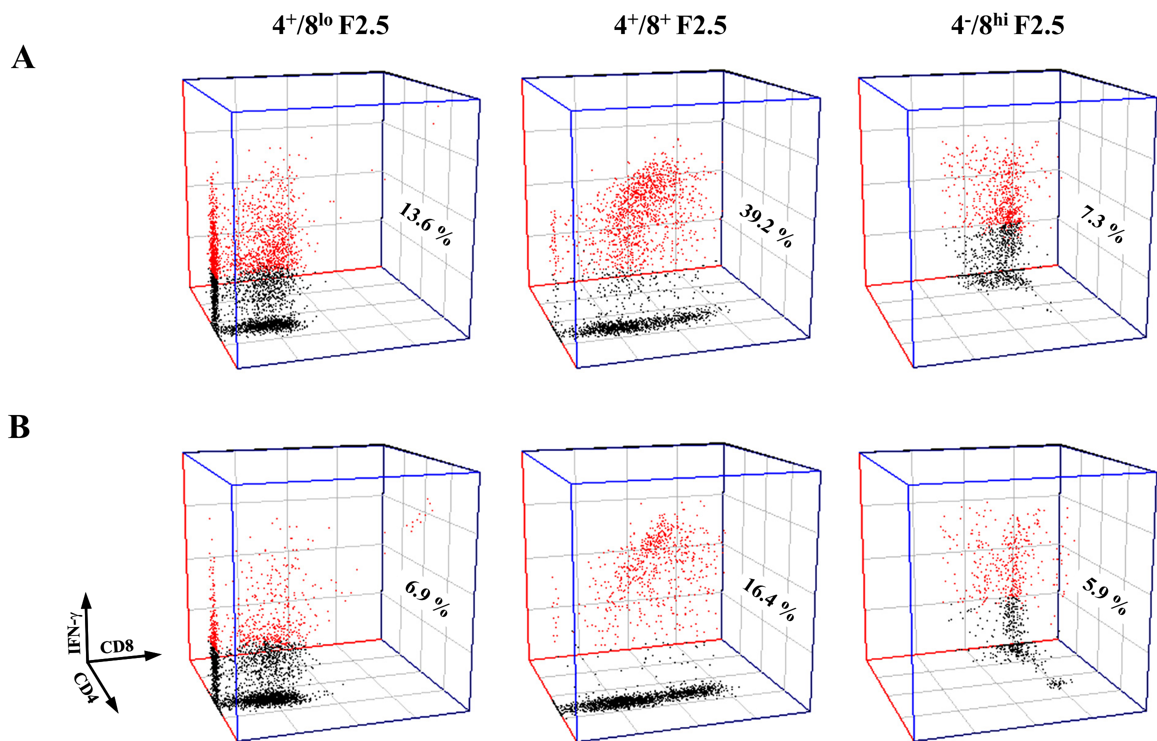

FIGURE 23: HER2-specific IFN-γ release by the F2.5 T cell clone isoforms after stimulation with NIH3T3 cell lysate-pulsed immature Mo-DC. 1 x 10^5 cells of the F2.5 T cell clone isoforms were stimulated with 1 x 10^4 immature Mo-DC loaded with NIH3T3^{HER2} or NIH3T3^{wt} whole cell protein extract. After 18 h of culture, cells were subjected to IFN-γ secretion analysis and counterstained with the CD4-specific mAb 13B8.2 and the CD8-specific mAb B9.11. IFN-γ secretion profile of the $4^+/8^{lo}$, $4^+/8^+$ and $4^-/8^{hi}$ F2.5 T cell clone isoforms in response to (**A**) NIH3T3^{HER2} cell lysate and (**B**) NIH3T3^{wt} cell lysate. In the three-color dot plots, expression of CD8 is given on the x-axis, IFN-γ on the y-axis and CD4 on the z-axis. The percentages of IFN-γ secreting cells (red) were estimated on the basis of a discriminating gate. Values are displayed in the plots.

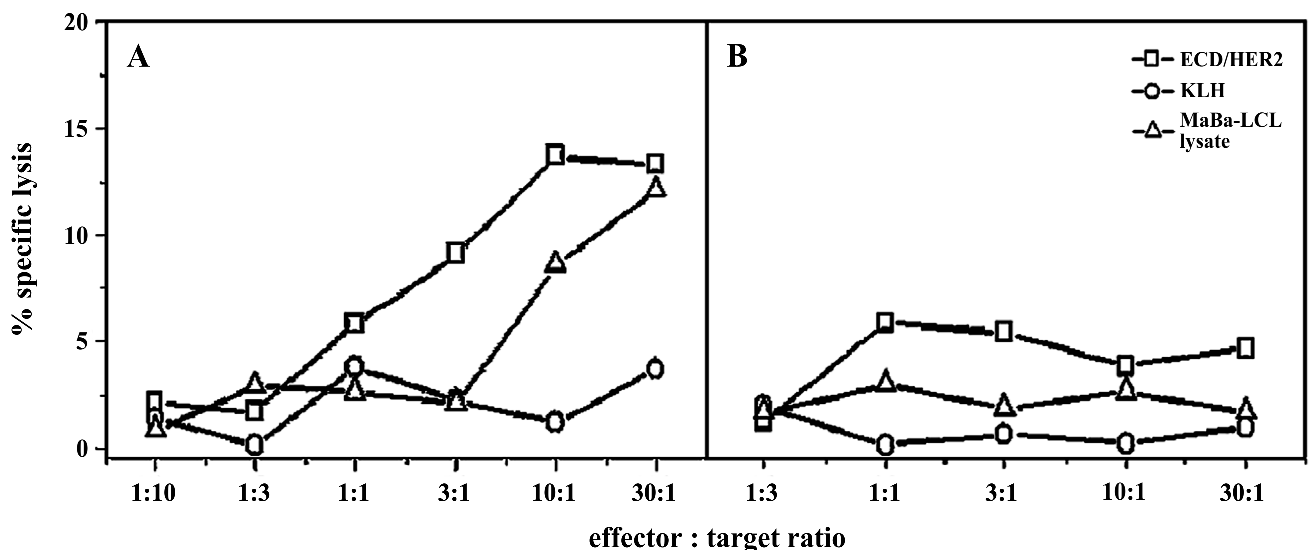

FIGURE 24: CTL activity of $4^-/8^{hi}$ and $4^+/8^+$ F2.5 T cells. The ability of $4^-/8^{hi}$ and $4^+/8^+$ F2.5 T cells to lyse autologous Mo-DC loaded with ECD/HER2, KLH or lysate of MaBa-LCL cells was determined in a standard ^{51}Cr release assay as described in material and methods. Cytolytic activities of (**A**) $4^-/8^{hi}$ F2.5 T cells and (**B**) $4^+/8^+$ F2.5 T cells.

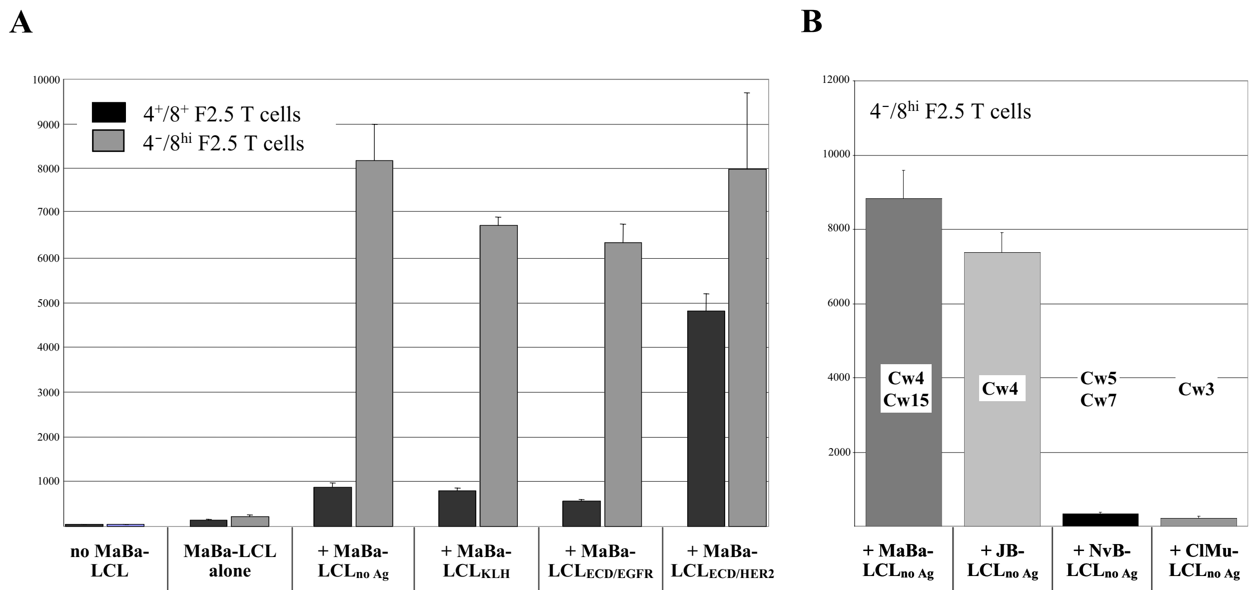

FIGURE 25: Proliferation of $4^+/8^+$ and $4^-/8^{hi}$ F2.5 T cells in coculture with autologous or allogeneic LCL. 1×10^4 T cells were incubated with 1×10^3 γ-irradiated LCL targets in U-bottom 96-well plates for 5 d with 1 µCi [^{3}H]TdR added during the last 16 h. Cells were harvested onto filter plates and the incorporation of radioactivity was estimated in a β-scintillation counter. (**A**) F2.5 T cell proliferation in response to different soluble protein antigens presented by MaBa-LCL cells. **Black bars**, $4^+/8^+$ F2.5 T cells; **grey bars**, $4^-/8^{hi}$ F2.5 T cells. (**B**) Proliferative reactivity of $4^-/8^{hi}$ F2.5 T cells to the allogeneic LCL cell lines JB (Cw4), NvB (Cw5/Cw7) and ClMu (Cw3). The levels of [^{3}H]TdR incorporation were compared with the levels observed in response to MaBa-LCL cells. Controls for each assay included F2.5 T cells alone and γ-irradiated LCL alone. No significant [^{3}H]TdR uptake was observed in any of these controls, thus they were omitted in panel B for sake of clarity. Results are expressed as the mean of total cpm ± SD of quadruplicate cultures.

5.14. $4^-/8^{hi}$ F2.5 T cells elicit cytolytic and proliferative responses to MaBa-LCL in the absence of HER2 antigen

HER2-induced cytolytic activity of $4^-/8^{hi}$ and $4^+/8^+$ F2.5 T cells was evaluated in a standard ^{51}Cr release assay with antigen-loaded autologous DC as target cells. This was performed according to the concept that the major attribute of activated $CD8^+$ T lymphocytes is the ability to kill cells presenting immunogenic peptides in complex with MHC class I molecules. As presented in Fig. 24, T cells of the $4^-/8^{hi}$ F2.5 isoform exhibited weak cytolytic activity against ECD/HER2-loaded DC. However, cytotoxicity occurred also against DC loaded with whole protein extract of MaBa-LCL, albeit at a higher effector-to-target cell ratio. KLH-loaded DC served as a control and failed to provoke a measureable response. As shown in the same figure, $4^+/8^+$ F2.5 T cells did not exhibit any cytolytic activity against DC targets neither loaded with ECD/HER2 protein nor with MaBa-LCL lysate.

Reactivity of the $4^-/8^{hi}$ F2.5 T cells against MaBa-LCL cells was confirmed by standard proliferation assays. As presented in Fig. 25A, these analyses revealed the same functional differences between the two F2.5 T cell subsets. Whereas $4^+/8^+$ F2.5 T cells demonstrated HER2-spe-

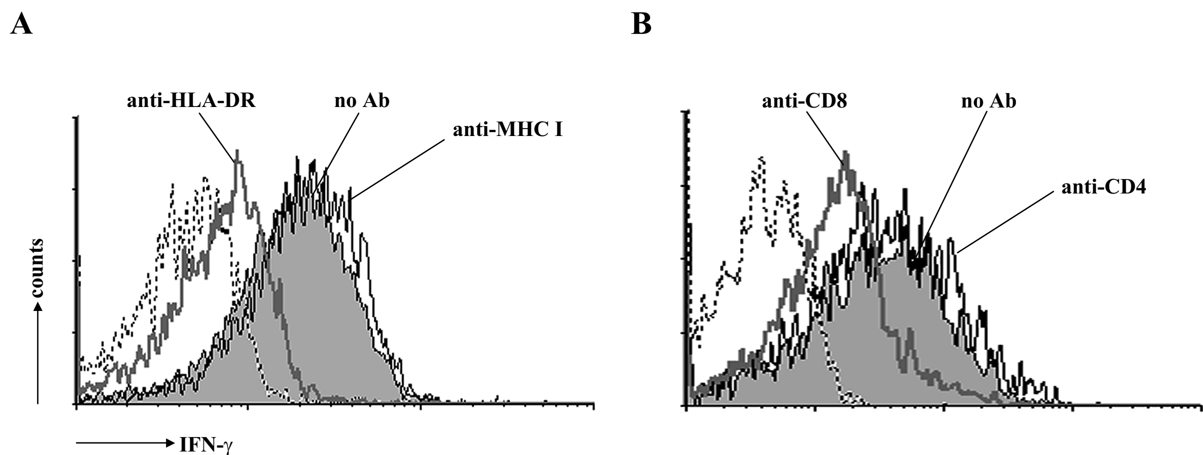

FIGURE 26: Effects of antibody-mediated MHC and coreceptor blockade on the expression of IFN-γ by ECD/HER2-stimulated $4^{-}/8^{hi}$ F2.5 T cells. For functional analysis, $4^{-}/8^{hi}$ F2.5 T cells were cocultured for 18 h with ECD/HER2-loaded immature Mo-DC in the presence or absence of 10 µg/ml of the indicated mAb's. Subsequently, cells were assayed for IFN-γ secretion by flow cytometry. Histogram overlay plots showing the effect of mAb-mediated inhibition of (**A**) MHC binding and (**B**) coreceptor function on the ECD/HER2-specific IFN-γ production of $4^{-}/8^{hi}$ F2.5 T cells.

cific proliferation by the detection of at least 5-fold higher [^{3}H]TdR incorporation rates in response to ECD/HER2-loaded MaBa-LCL as compared to the controls, the $4^{-}/8^{hi}$ F2.5 T cells exhibited enhanced proliferation to all APC-presented antigens.

Proliferation analysis with unloaded LCL of different haplotypes suggested, that $4^{-}/8^{hi}$ F2.5 T cells recognize antigen in a HLA-Cw4 restricted manner. As shown in Fig. 25B, incubation of the T cells with the HLA-Cw4 homozygous LCL line JB resulted in a [^{3}H]TdR uptake, that was in the same range as that observed for MaBa-LCL targets. In contrast, the LCL lines derived from the donors NvB and ClMu with the HLA-C haplotypes HLA-Cw5^{+}/-Cw7^{+} and HLA-Cw3$^{+/+}$ did not induce any significant proliferation.

In summary, the results indicate that $4^{-}/8^{hi}$ F2.5 T cells expressed two distinct specificities, the one against HLA-Cw4^{+} LCL target cells inducing proliferative and moderate cytolytic activity and the other against the HER2 protein antigen presented on autologous immature DC leading to the production and release of IFN-γ.

5.15. ECD/HER2-specific IFN-γ secretion of $4^{-}/8^{hi}$ F2.5 T cells depends on HLA-DR and CD8

Antibody-blocking assays were performed on the $4^{-}/8^{hi}$ F2.5 T cell clone isoform to elucidate the mechanisms underlying this unusual phenotypic transition and degenerate antigen recognition pattern. The mAb's W6/32 specific for MHC class I molecules, HB55 for HLA-DR, anti-CD4 for CD4 and RIV II for CD8, were chosen for this kind of functional analysis, since their blocking activities are well documented (Tomkinson B.E. et al., 1989; Tüting T. et al., 1998; Piatier-Tonneau D., 1998). In the study, the antibodies were added to mixed cultures of $4^{-}/8^{hi}$

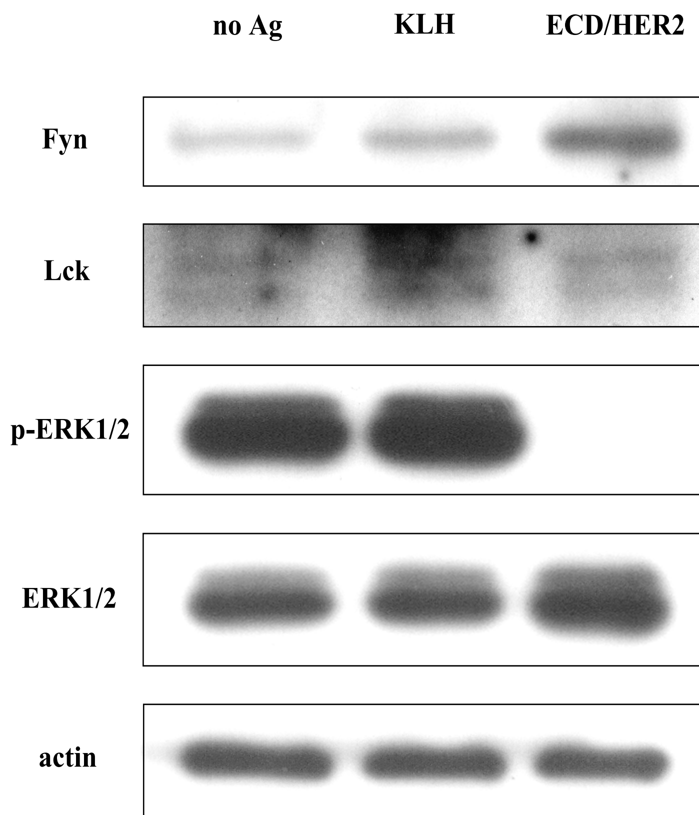

FIGURE 27: Relative expression of the Src kinases Fyn and Lck and phosphorylation status of ERK 1 and 2 in cocultures of $4^{-}/8^{hi}$ F2.5 T cells with antigen-loaded immature Mo-DC. Cell lysates prepared from 20 h cocultures of 1 x 10^6 T cells and 2 x 10^4 antigen-loaded immature DC were subjected to electrophoresis on a 7.5% SDS-PAGE and transferred to a PVDF membrane. The blot was first immunostained with mAb E10, recognizing Thr-202 and Tyr-204 dually phosphorylated ERK 1 and 2. Using the same blot after stripping of bound Ig, the amount of total ERK was determined using rabbit anti-p44/42 MAP kinase pAb. Next, expression of Fyn was evaluated with the goat pAb FYN-3. This was followed by the detection of Lck using the mouse mAb 3A5. To confirm equal loading the membrane was finally reprobed with mouse mAb JLA, an IgM specific for actin.

F2.5 T cells and ECD/HER2-loaded immature DC and analyzed for their ability to inhibit antigen-induced IFN-γ secretion. As shown in Fig. 26A, the frequency of IFN-γ secreting cells was markedly reduced in response to HB55, but was completely unaffected to W6/32 treatment. The result indicated that although CD4 was absent on these effector T cells, ECD/HER2 antigen presentation was restricted by HLA-DR. This obviously raised the question of whether the TCR signals initiated by pMHC class II binding were enhanced by CD8 coreceptor ligation. A positive effect of CD8 on the activation status of $4^{-}/8^{hi}$ F2.5 T cells was evidenced due to the finding that the ECD/HER2-specific IFN-γ production could be inhibited by the addition of the CD8-specific mAb RIV II (Fig. 26B). As a control, treatment with the anti-CD4 mAb had no effect on the IFN-γ expression levels.

5.16. ECD/HER2-specific $4^{-}/8^{hi}$ F2.5 T cell responses are associated with increased Fyn expression and inhibition of ERK phosphorylation

Immunoblot analyses were performed on whole cell extracts derived from cocultures of $4^{-}/8^{hi}$ F2.5 T cells with autologous immature Mo-DC that were either unloaded or loaded with the antigens KLH or ECD/HER2 in order to determine which components of the signal transduction process became specifically activated during ECD/HER2-driven T cell activation. Fig. 27 summarizes the results obtained by sequential staining of an immunoblot with mAb's specific for the antigens indicated on the left margin. As depicted in this figure, stimulation of $4^{-}/8^{hi}$ F2.5 T cells by ECD/HER2-loaded DC led to a marked upregulation of Fyn, a Src family protein tyrosine

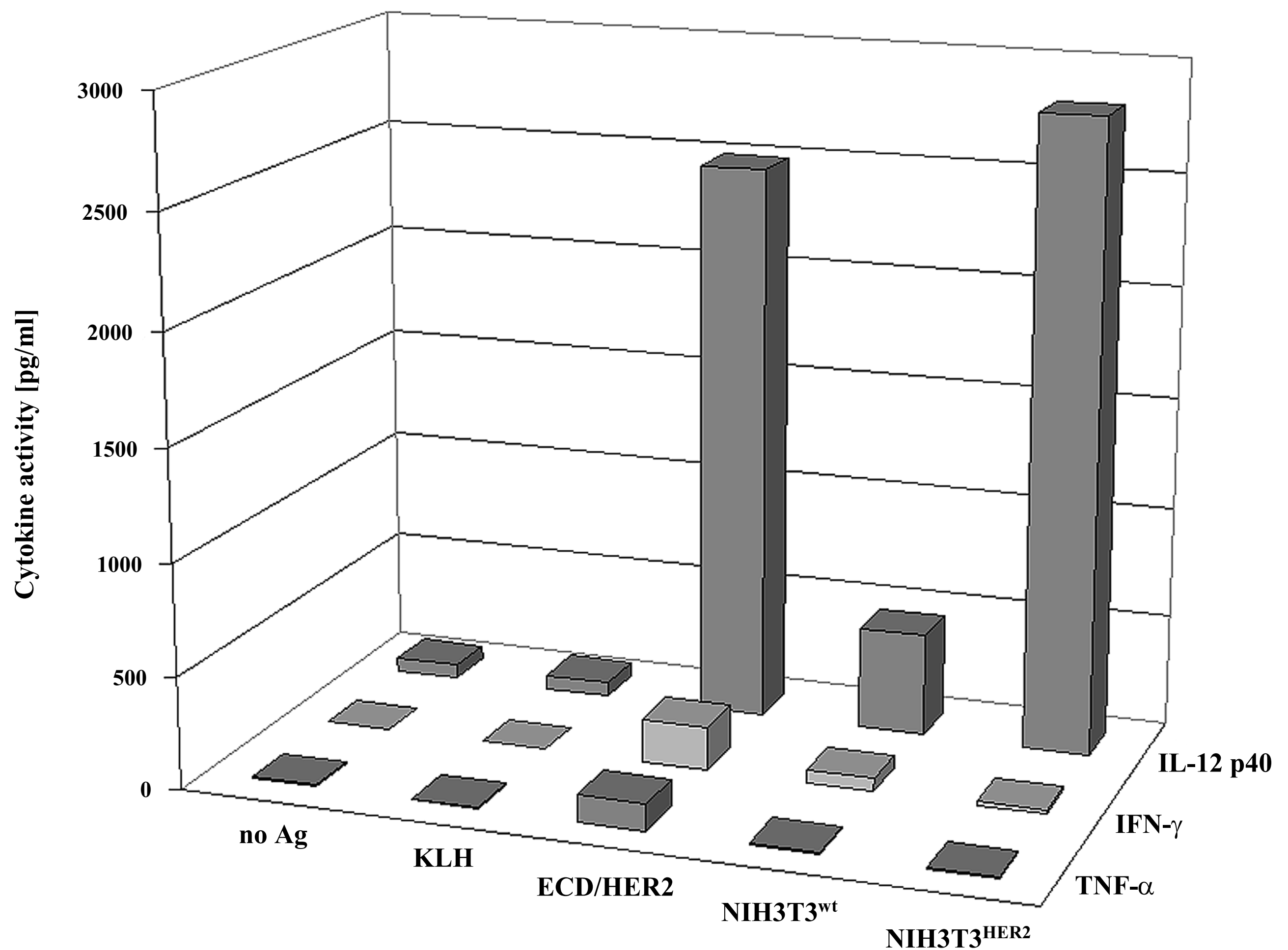

FIGURE 28: Cytokine production in cocultures of $4^{-}/8^{hi}$ F2.5 T cells and immature Mo-DC loaded with protein antigens or NIH3T3 cell lysates. 1 x 10^5 $4^{-}/8^{hi}$ F2.5 T cells were stimulated with 1 x 10^4 immature Mo-DC loaded with the indicated antigens in the wells of 96-well plates. Supernatants were collected at 20 h and assayed for IL-12 p40, IFN-γ and TNF-α production by ELISA. Results are expressed as pg/ml. The bars shown represent the mean of triplicate cultures from one of two independent experiments. Error bars were <5% of the mean and are omitted for sake of clarity.

kinase that can partially inhibit TCR-mediated T cell activation (Gajewski T.F. et al., 1995; Yasuda K. et al., 2002). In contrast to Fyn, extraordinary low levels of Lck, a second Src family member associated with the TCR/CD3 complex (Zamoyska R. et al., 2003), were observed in all three $4^{-}/8^{hi}$ F2.5 T cell cultures. Absence of reactivity was not due to inefficient binding of the mAb, since Lck could be readily detected in lysate from unstimulated T cells of donor MaBa (data not shown). Furthermore, the culture was characterized by an almost complete absence of Thr-202 and Tyr-204 phosphorylation of the MAP kinases ERK 1 and 2. Dephosphorylation rather than a decrease in protein expression seemed to be responsible for the inhibition of ERK signaling since a mAb recognizing both phosphorylated and unphosphorylated forms confirmed equivalent amounts of these kinases in each lane. Equal total protein loading of the cell extracts in the different lanes was verified by reprobing the blot with an actin-specific mAb.

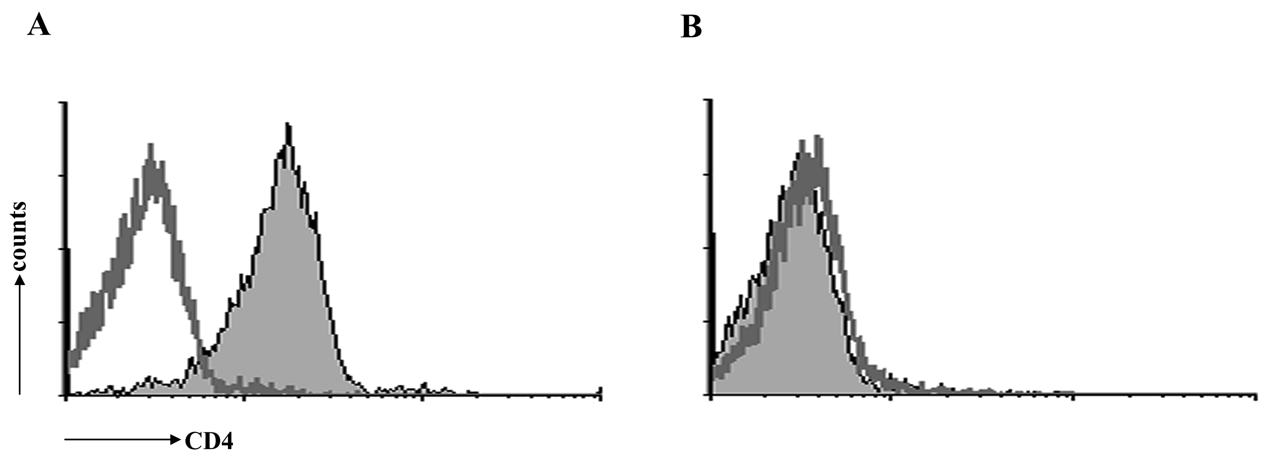

FIGURE 29: Induction of CD4 expression on $4^-/8^{hi}$ F2.5 T cells after exposure to exogenous IL-12. $4^-/8^{hi}$ F2.5 T cells were cocultured for 16 d with ECD/HER2-loaded γ-irradiated MaBa-LCL as APC in the presence and absence of 0.2 ng/ml IL-12. Subsequently, CD4 expression on viable (PI^-) CD8-gated $4^-/8^{hi}$ F2.5 T cells was determined in a flow cytometric analysis using the CD4-specific mAb 13B8.2 and the CD8-specific mAb B9.11 for cell staining. Histograms for $4^-/8^{hi}$ F2.5 T cell cultures (**A**) in TZM with 0.2 ng/ml IL-12 and (**B**) in TZM without exogenous IL-12.

5.17. Continuous administration of IL-12 to $4^-/8^{hi}$ F2.5 T cells results in CD4 expression and induces apoptosis

Expression levels of TNF-α, IFN-γ and IL-12 p40 in supernatants derived from cocultures of $4^-/8^{hi}$ F2.5 T cells and antigen-loaded immature Mo-DC were determined by ELISA. As shown in Fig. 28, moderate amounts of TNF-α and IFN-γ were found in response to ECD/HER2, but not to the other antigens studied. In accordance to the flow cytometry-based IFN-γ secretion analysis presented in Fig. 23, ELISA testing did not detect significant levels of IFN-γ in the cultures with $NIH3T3^{HER2}$ extract as the antigen. In contrast to the effector T cell cytokines, IL-12 p40, secreted mainly by CD40-activated DC (Kelsall B.L. et al., 1996; Kato T. et al., 1997), was strongly upregulated in response to both ECD/HER2 protein and lysate from $NIH3T3^{HER2}$ cells. Since antigen-loaded DC alone did not produce any detectable amounts of IL-12 p40 (data not shown), an inductive signal for its expression had to be provided by the activated $4^-/8^{hi}$ F2.5 T cells.

Evidence that IL-12 had significant impact on the differentiation of the F2.5 T cell clone was given by a suspension culture of $4^-/8^{hi}$ F2.5 T cells that was treated with 0.2 ng/ml of recombinant IL-12. In a flow cytometric analysis, that was performed 2 weeks after the first addition of IL-12, a marked decrease in viable cell counts was observed since only 28% of the cells were negative for PI compared to 64% under standard culture conditions. Surprisingly, as shown in Fig. 29, the residual viable cells of the IL-12-treated culture exhibited strong staining for CD4. This result indicated that high levels of IL-12 induced CD4 expression, but also sensitized $4^-/8^{hi}$ F2.5 T cells to apoptosis.

6. Discussion

6.1. Different HER2 antigen sources and appropriate specificity controls are indispensable for the selection of HER2-specific CD4$^+$ T cells from human blood

The aim of this study was the generation and maintenance of HER2-specific CD4$^+$ T cell lines and clones using *in vitro* generated autologous DC and LCL as APC. Since the native HER2 protein was used as a target antigen only weak T cell responses were assumed due to deletion or anergy induction of highly autoreactive T cells in the thymus (Kappler J.W. et al., 1987; Ramsdell F. et al., 1989). As a consequence, highly purified protein as the source of antigen and potent professional APC for efficient antigen uptake and MHC class II-restricted presentation was required to overcome T cell tolerance to HER2 in an *in vitro* culture system.

Due to these requirements, the first aim of this study was to establish a method for the efficient production of pure recombinant ECD/HER2 protein in a CHO cell expression system. As a result of a high-density cell culture in a modular minifermenter under serum- and protein-free conditions ECD/HER2 was the only protein present in elevated amounts in the culture medium. Consequently, by using transversal dialysis and IMAC chromatography it could be purified to almost complete homogeneity.

In a recently published attempt to generate TAA-reactive CD4$^+$ T cells by *in vitro* culture with recombinant MAGE-3 protein as the antigen, Chaux P. et al. (1999) noted that a large number of the CD4$^+$ clones obtained were directed against contaminants of the protein batch. Due to this finding, further antigen preparations were established to prove HER2-specificity of the *in vitro* generated Th cell lines and clones. First, another batch of ECD/HER2 protein was available being produced in a mouse L cell expression system. T cell reactivity to both of these antigens was taken as a strong indication for HER2-specificity, since the two ECD/HER2-proteins were purified by different chromatographic methods. Secondly, for the identification of T cells that discriminate between ECD/HER2 and ECD/EGFR, the latter was produced in CHO cells and used as a negative specificity control. Other negative controls included KLH as a well-defined highly immunogenic soluble antigen and FCS as a complex mixture of xenogenic protein antigens. Finally, whole protein extract from NIH3T3^{HER2} cells containing substantial amounts of HER2, was used as a source of antigen in order to test reactivity of ECD/HER2-responsive T cells under conditions similar to those at the site of tumor growth. This was considered feasible, since immature DC were able to engulf particulate material, such as apoptotic cells and microorganisms (Nouri-Shirazi M. et al., 2000). One further advantage of this NIH3T3 cell-based screening method was that lysate from NIH3T3^{wt} cells could serve as a very stringent negative control.

6.2. The *in vitro* generated Mo-DC efficiently process purified erbB antigens and after maturation express a complete set of costimulatory molecules

DC were generated from blood monocytes of donor MaBa under FCS-free conditions in the pres-

ence of IL-4 and GM-CSF according to the protocol by Jonuleit H. et al. (1997). Under these particular culture conditions cells lost their ability to adhere to plastic and became $CD14^{-}CD83^{lo}$ indicating an immature DC phenotype (Zhou L.J. and Tedder T.F., 1995). Since immature DC are characterized by a high ability of antigen uptake (Banchereau J. and Steinman R.M., 1998; Cella M. et al., 1997), cells were exposed to soluble protein antigens or extract of lysed cells at this stage. The immature DC were able to endocytose high amounts of protein added to the culture medium, as could be demonstrated by immunofluorescence microscopy.

PNGase F treatment of recombinant ECD/HER2 and ECD/EGFR revealed that both proteins are highly N-glycosylated since their apparent molecular masses in SDS-PAGE decreased from 105 to 75 kDa following digestion. As reported by Hiltbold E.M. et al. (2000), an effect of protein glycosylation may be protection of the antigen against endosomal degradation. This was attributed to selective uptake by the mannose receptor (CD206) and retention in early endosomes. In striking contrast to these authors, rapid internalization of ECD/EGFR protein in a mannose receptor-independent fashion was observed in the present study. The ingested protein was rather transported in small vesicles to perinuclear located $LAMP\text{-}1^{+}$ late endosomes and HLA-DR-rich MIIC compartments thus providing evidence that uptake occurred mainly by macropinocytosis. The capability to form pinocytic vesicles was reported by Sallusto F. et al. (1995) to be a very efficient mechanism of immature DC to capture small particles and soluble antigens present in the extracellular fluid. Most importantly, its well defined colocalization with HLA-DR suggests that ECD/EGFR as an exogenous glycosylated protein was properly processed in the MHC class II pathway for antigen presentation to $CD4^{+}$ T cells.

After antigen loading, DC used for the priming of $CD4^{+}$ T cells were matured with a cocktail of proinflammatory cytokines and PGE_2 (Jonuleit H. et al., 1997). High levels of CD83 expression indicated the successful differentiation to the fully mature state. Moreover, the cells displayed increased surface expression of HLA-DR indicating the formation of large amounts of pMHC class II complexes. The mature DC also expressed various accessory molecules important for enhanced signaling and adhesion. Most notably were the intense expression of the B7 activation markers CD80 and CD86 which reflected their high T cell stimulatory capacity. Because TCR engagement without CD28-B7 interaction induce anergy in naive T cells (Yi-qun Z. et al., 1997; Appleman L.J. and Boussiotis V.A., 2003), only mature DC were used as APC in the primary cultures to achieve full T cell activation. Another feature of the cells was a Th1-promoting activity indicated by the expression of the adhesion molecule CD54, which strongly interacts with the LFA-1 receptor on T cells (Smits H.H. et al., 2002). Th1 polarization is another essential step in the induction of antitumor immunity, since the cells stimulate macrophage and CTL activity by secretion of IL-2, IFN-γ and TNF-α (Swain S.L., 1995).

As reviewed by Grewal I.S. and Flavell R.A. (1998), CD40-CD40L interactions are critical for the elicitation of Th1 cell-dependent effector functions. Since almost all ECD/HER2-loaded DC, but only about half of the $NIH3T3^{HER2}$ lysate-loaded DC expressed the CD40 receptor on their surface, the former cells were used for the induction of primary T cell responses. Different endocytotic pathways for soluble antigens and fragments of dead cells may account for the observed differences in

CD40 expression. Sauter B. et al. (2000) gave evidence that DC can even discriminate between apoptotic and necrotic cells, adversely affecting their maturation and function.

6.3. Stimulation of $CD4^+$ T cell microcultures with ECD/HER2-loaded autologous DC provides an applicable method for the generation of HER2-reactive Th cell lines

For the generation of HER2-specific Th cell lines, freshly isolated $CD4^+$ T cells from donor MaBa were stimulated with ECD/HER2-loaded autologous mature DC. Massive cell death, however, occurred in the first two weeks of *in vitro* culture. During this critical period, T cells were maintained in high-density microcultures to facilitate exchange of soluble factors and the formation of cell-cell contacts. Furthermore, IL-2 was added to the cultures as it promotes proliferation of activated T cells (Smith K.A., 1988), as well as IL-7 due to its ability to inhibit apoptosis (Vella A.T. et al., 1998; Boise L.H. et al., 1995) and to provide costimulation (Chazen G.D. et al., 1989; Costello R. et al., 1993). For the inhibition of CD152 expression on primed T cells that may act as a negative feedback mechanism to limit T cell activation (Finn P.W. et al., 1997), immature DC with low levels of CD80 and CD86 were used as APC for all subsequent weekly restimulations. Such an approach was thought to be suitable due to the finding that previously activated T cells no longer require the costimulatory signal transduced by CD28 for their clonal expansion (Peterson K.E. et al., 1999). As a result of all these efforts, clusters of proliferating T cells could be observed in some of the microcultures between the first and second restimulation.

HER2-specificity of the proliferating T cell microcultures was evaluated by detecting IFN-γ release in response to processed ECD/HER2 soluble antigen and whole cell lysate of $NIH3T3^{HER2}$ fibroblasts on immature DC. In order to prevent selection of T cells directed against contaminants in the protein preparation or culture medium, only T cell microcultures that displayed reactivity to both antigen sources were considered to be HER2-specific and selected for the subsequent cloning and expansion steps. This stringent strategy was chosen due to the expectation that $CD4^+$ T cells readily react to virtually any foreign antigens while avoiding immune responses to self, although the ECD/HER2 protein was produced in an eukaryotic expression system and found to be LPS-free by the Limulus amebocyte assay.

The production of IFN-γ was first assessed by ELISPOT analysis. This technique allows rapid and reliable screening with low cell numbers and therefore could be applied at a time when cell proliferation was first observed by light microscopy. By use of this technique, strong HER2-reactivity could only be demonstrated for one microculture termed F2. A subsequently performed flow cytometry-based IFN-γ secretion analysis with concomittant detection of CD4 and CD8 coreceptor expression demonstrated the existence of two different subsets of IFN-γ producing T cells in the F2 T cell line. The one displayed a $CD4^-CD8^+$ and the other a $CD4^+CD8^{lo}$ T cell phenotype. Surprisingly, both cell types were reactive against HER2 antigen. Since $CD4^+$ sorted T cells were used to establish HER2-specific T cell lines, $CD8^+$ T cells were likely contaminants of either the $CD4^+$ T cell fraction or of one of the monocyte preparations used to generate ECD/HER2-loaded DC. By flow cytometric analysis it was found that the Mo-DC cultures contained few numbers of contaminating lymphocytes, main-

ly B cells and neutrophils, but also T and NK cells. Since these cultures were essential for further expansion of the F2 T cell line, it was of particular interest to establish a fast and reliable method that allows the discrimination of HER2-specific $CD4^+$ F2 T cells from other cells.

A major consequence of clonal selection and expansion in response to periodic antigen exposure is the change of the TCR repertoire from a polyclonal to a more restricted pattern (Boitel B. et al., 1992). Therefore, the most reliable way for the successful detection of HER2-reactive T cells without knowledge of the peptide epitope is to screen the F2 T cell line for predominant TCR Vβ utilization. Flow cytometric analysis with a panel of mAb's covering approximately 60% of the TCR Vβ repertoire revealed that 85% of the $CD4^+$ T cells in the F2 T cell line expressed TCR Vβ5.3. As compared to freshly isolated $CD4^+$ T cells of donor MaBa, this represents an approximately 80-fold increase in relative cell numbers. In contrast, preferential TCR Vβ usage could not be identified for the $CD4^-CD8^+$ F2 T cell subset. Since Akbar A.N. et al. (1988) demonstrated that naive T cells switch from CD45RA to CD45RO expression upon activation, the detection of high levels of CD45RO, but not CD45RA on the $CD4^+CD8^{lo}$TCR $V\beta5.3^+$ F2 T cells provided further evidence for an antigen-driven selection process.

6.4. Antigen-specific stimulation with MaBa-LCL cells results in selection of the apoptosis-resistant T cell clone F2.5

Due to their restricted expression of TCR Vβ5.3, ECD/HER2-specific $CD4^+CD8^{lo}$ F2 T cells were sorted by MACS using anti-TCR Vβ5.3 mAb-labeled immunomagnetic beads before limiting dilution cloning. After two weeks of culture with ECD/HER2-pulsed γ-irradiated MaBa-LCL as stimulator and feeder cells, 12 out of 120 cultures contained proliferating T cells. All T cell clones showed an identical expression pattern for CD4, CD8 and TCR Vβ5.3, indicating that they were derived from the same precursor cell. 11 of these clones could not be maintained in culture and underwent apoptosis, whereas one clone, designated as F2.5, depicted good viability and proliferated upon ECD/HER2-specific stimulation.

To assure viability of F2.5 T cells in the subsequent antigen-specific expansion steps, T-Stim, a conditioned medium from PHA-stimulated human PBMC cultures, was added to the culture medium together with IL-2 and IL-7. The finding that F2.5 T cells could not be maintained in culture for more than two weeks in the absence of MaBa-LCL cells indicated that proliferation was not affected by the medium supplements. Moreover, based on the observation that treatment of F2.5 T cells with the TCR Vβ5.3-specific mAb 3D11 provoked a strong IFN-γ response, growth in 3D11-coated cell culture plates provided an alternative method of T cell expansion in the absence of APC.

However, under both culture conditions, T cells exhibited profound phenotypic changes. In the presence of ECD/HER2-loaded MaBa-LCL cells the F2.5 T cells completely lost the expression of CD4 and became CD8 bright positive, whereas incubation with the 3D11 mAb led to the development of $CD4^+CD8^+$ DP cells with heterogenous CD8 expression levels. In contrast to the parental $4^+/8^{lo}$ F2.5 T cells, these $4^-/8^{hi}$ and $4^+/8^+$ isoforms could be grown stably in culture for more than 6 months.

6.5. The F2.5 T cell clone adopts a CD8 memory phenotype during culture with ECD/HER2-loaded MaBa-LCL cells

Flow cytometric analysis found that $4^{-}/8^{hi}$ F2.5 T cells expressed CD8α and CD8β whereas $4^{+}/8^{+}$ F2.5 T cells showed only CD8α. Mainly by the exclusive presense of CD8α it was suggested that $4^{+}/8^{+}$ F2.5 T cells represented a transient intermediate on the way to a memory phenotype. This is supported by studies of Madakamutil L.T. et al. (2004) who found an upregulation of the cytokine receptors IL-7Rα and IL2/IL15Rβ on $CD8\alpha^{+}\beta^{-}$ T cells upon activation. The essential role of these two cytokine receptors in memory T cell development is demonstrated by their ability to increase expression of the survival factors Bcl-xL and Bcl-2 (Kim S.V. and Flavell R.A., 2004). On the other side, concomitant expression of CD8α and CD8β results in the formation of both CD8 homo- and heterodimers on $4^{-}/8^{hi}$ F2.5 T cells. As reported by several authors (Renard V. et al., 1996; Bosselut R. et al., 2000; Wong J.S. et al., 2003) CD8β generally increases the avidity of CD8 binding to MHC molecules and mediates enhanced interaction with the TCR/CD3 complex.

Further evidence for the conversion to a memory phenotype was obtained by two additional findings. First, detection of a significant degree of CD27 surface expression on $4^{-}/8^{hi}$ F2.5 T cells. A benefical role of the CD27 costimulatory receptor in the formation and the persistance of a memory T cell pool was suggested, due to its ability to counteract apoptotic signals and to enhance TCR-induced expansion by decreasing the threshold for T cell activation (Hendriks J. et al., 2000). Secondly, detection of KIR 2DL2 on approximately 20% of the $4^{-}/8^{hi}$ F2.5 T cells. According to Mingari M.C. et al. (1996), suggesting KIR expression on T cells being restricted to oligoclonally or monoclonally expanded $CD8^{+}$ T cells, this was taken as an additional indication of memory T cell development.

6.6. The thymocyte-like phenotype of the F2.5 T cell clone provides evidence of escape from central tolerance by its precursor cell

The way F2.5 T cells changed their phenotype bears some analogy to CD4/CD8 lineage decision in the thymus. Therefore, the most consistent explanation for the induction of F2.5 T cell differentiation into the CD8 lineage is that the precursor cell of the F2.5 clone escaped as a low affinity autoreactive T cell negative selection and persisted in the periphery in an uncommited state, apparently ignorant of endogenous HER2. Evidence for an escape of low affinity self-reactive T cells from thymic negative selection was first provided by functional characterization of myelin proteolipid protein-specific T cells in an experimental autoimmune encephalomyelitis mouse model (Liu G.Y. et al., 1995; Anderson A.C. et al., 2000).

Whereas the classical instruction and stochastic models of lineage commitment are based on the central paradigm that lineage commitment occurs in DP thymocytes (von Boehmer H. and Kisielow P., 1993; Germain R.N., 2002; Singer A. et al., 1999), the currently most favored model, known as

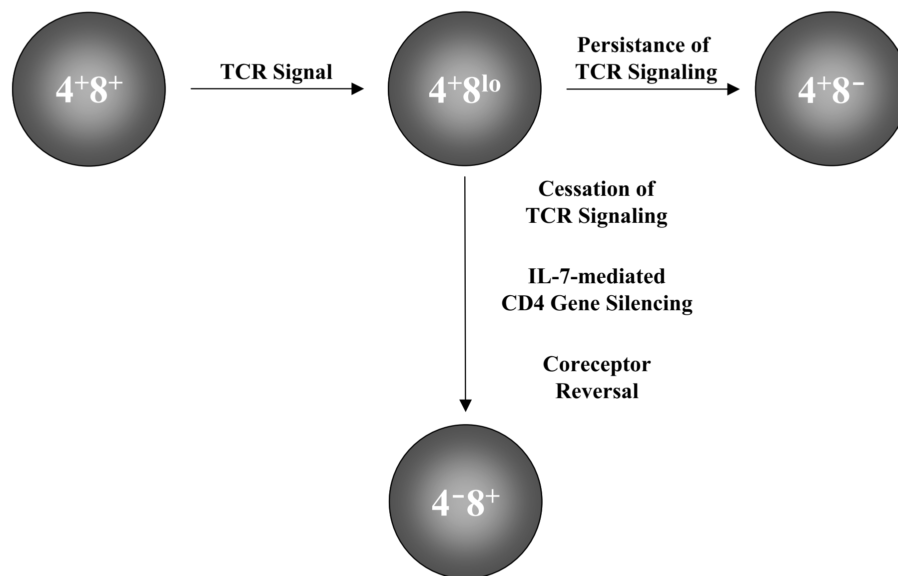

FIGURE 30: Schematic representation of the kinetic signaling model of T cell development. The model postulates that TCR signals drive CD4$^+$CD8$^+$ DP thymocytes to differentiate into transitional CD4$^+$CD8^{lo} cells. At this critical stage of development, CD4/CD8 lineage decision is determined by whether TCR signals are still present or have ceased. Persistance of TCR signaling promotes CD4 lineage commitment. In contrast, TCR disengagement of MHC class I ligands caused by diminished CD8 surface levels results in cessation of TCR signaling and coreceptor reversal. This process is further enhanced by CD4 gene silencing in response to IL-7. As a result, thymocytes differentiate into mature CD8 SP T cells.

the kinetic signaling model of T cell development, proposes that CD4$^+$CD8^{lo} transitional cells are located at this developmental branch point (Singer A., 2002; Bosselut R. et al., 2003). In this regard, the latter model assumes that MHC class I-signaled thymocytes differentiate into CD8$^+$ T cells because of cessation of TCR signaling caused by a diminished CD8 surface expression of DP thymocytes. The absence of TCR signaling leads to silencing of CD4 and induction of CD8 gene expression, in this model referred to as coreceptor reversal (Brugnera E. et al., 2000). Starting from CD4$^+$CD8^{lo} T cells in the original F2 T cell line, the course of F2.5 T cell differentiation into CD8 SP T cells is in line with the predictions of this model for the lineage commitment to a CD8 SP phenotype, as presented schematically in Fig. 30.

6.7. Increased responsiveness to IL-2 and IL-7 may promote the development of the F2.5 T cell clone into the CD8 SP lineage

Resting T cells are negative for HLA class II antigens but may initiate expression upon persistent stimulation (Ferenczi K. et al., 2000). Therefore, flow cytometric detection of high levels of HLA-DR on all three F2.5 T cell clone isoforms indicated that T cells had entered a state of sustained activation and proliferation under the chosen culture conditions. However, the medium supplements IL-2, IL-7

and T-Stim seemed to be crucial for the maintenance of this stable activated phenotype, since attempts to omit one of these additives resulted in a rapid loss of cell viability. Besides their prosurvival activity mediated by an upregulation of Bcl-2 via common cytokine receptor γ chain (γc) signaling (Nakajima H. and Leonard W.J., 1999), it was suggested that IL-2 and IL-7 also had a strong impact on the development of the F2.5 T cell clone. This was assumed due to the fact that F2.5 T cells exhibited thymocyte like characteristics and should therefore be susceptible to the γc-dependent signal transduction pathways that promote commitment to the CD8 phenotype (Paliard X. et al., 1988; He Y.W. et al., 1997). In particular, IL-7 appears to act as a master cytokine for CD8 T cell differentiation since it enhances CD4 gene silencing in DP thymocytes (Yu Q. et al., 2003). However, culture of F2.5 T cells on immobilized anti-TCR Vβ5.3 mAb 3D11 led to the generation of a stable $CD4^{+}CD8\alpha^{+}\beta^{-}$ DP phenotype, whereas culture with ECD/HER2-loaded MaBa-LCL resulted in a $CD4^{-}CD8\alpha\beta^{+}$ SP phenotype. Since both expansion strategies were performed in medium containing the same amounts of IL-2, IL-7 and T-Stim, additional signals provided by cognate interaction with the APC seemed to account for full differentiation to $CD8\alpha\beta^{+}$ SP T cells.

6.8. Coexpression of CD4 and CD8α and downregulation of CD5 may represent an adaptive response of $4^{+}/8^{+}$ F2.5 T cells to enhance TCR signaling

Despite the fundamental phenotypic changes, both $4^{-}/8^{hi}$ and $4^{+}/8^{+}$ F2.5 T cells secreted IFN-γ after ECD/HER2-specific stimulation. Pronounced differences in the degree of reactivity, however, were detected between the two isoforms. Whereas $4^{-}/8^{hi}$ F2.5 T cells showed the same extent of IFN-γ release as the parental $4^{+}/8^{lo}$ F2.5 T cells, $4^{+}/8^{+}$ F2.5 T cells exhibited a higher capacity for IFN-γ production. Two reasons may account for the increased responsiveness of the latter. First, more potent signaling due to concomitant MHC ligand binding of both CD4 and CD8α, and secondly, reduced expression of the immune inhibitory receptor CD5 (Tarakhovsky A. et al., 1995; Azzam H.S. et al., 2001). In respect to the role of CD5, Azzam H.S. et al. (1998) showing its level of expression on mature T cells being positively correlated with TCR signal intensity. In this study, high-level CD5 expression on $4^{+}/8^{lo}$ and $4^{-}/8^{hi}$ F2.5 T cells seemed to be a consequence of MHC-dependent antigen presentation, since $4^{+}/8^{+}$ F2.5 T cells, expanded in the absence of the HER2 antigen, expressed significantly lower CD5 levels. In this context, it is important to note that Hawiger D. et al. (2004) described a form of peripheral tolerance in which DC present the relevant antigen, but also transmit signals to induce strong CD5 expression on the responding T cells.

6.9. Antagonist peptide-mediated TCR signaling may explain MHC class II-dependent activation of $4^{-}/8^{hi}$ F2.5 T cells

The finding of $4^{-}/8^{hi}$ F2.5 T cells remaining capable of producing IFN-γ in response to ECD/HER2 presentation, raised the question whether F2.5 T cell reactivity was MHC restricted. To check this

issue, monoclonal antibody inhibition studies were performed on $4^{-}/8^{hi}$ F2.5 T cells in order to define the nature of the restriction element and the role of the CD8 coreceptor. The experiments clearly showed a selective inhibition of HER2-specific IFN-γ secretion by blocking mAb's specific for HLA-DR and CD8.

A possible explanation for the natural occurrence of such MHC class II-restricted $CD8^{+}$ T cells is provided in literature by studies focusing on TCR signaling in response to antagonist peptides. Antagonist peptides were originally described as altered peptide analogs, in which the native peptide is modified by amino acid substitutions at crucial contact sites for the TCR (Evavold B.D. et al., 1993; Kuchroo V.K. et al., 1994). Their unique property is the ability to prevent or modulate T cell activation induced by the stimulatory peptide. Several studies have indicated that antagonist peptides were also frequently found in the endogenous peptide pool (Klenerman P. et al., 1994; Khanna R. et al., 1996; Vidal K. et al., 1996; Carson R.T. et al., 1999). Of particular significance for their function is that antagonist peptides generally engage the TCR with lower avidity than their agonist analogues (Alam S.M. et al., 1996).

In a TCR transgenic mouse model, Volkmann A. et al. (1998) demonstrated CD8 differentiation of thymocytes specific for a MHC class II-restricted epitope of the complement factor C5 induced by an antagonist peptide. Since anti-MHC class II antibodies were able to block the generation of CD8 cells, it was suggested that this altered peptide variant was still presented on MHC class II molecules. An explanation for this uncommon feature, given by the authors, concerns the strength of signals transmitted through the protein tyrosine kinase Lck. Their line of argument emphasizes that weak binding of the TCR results in a higher off rate, which diminishes coreceptor binding and recruitment of Lck to the TCR/CD3 complex. The fact that Lck binds with higher affinity to CD4 than to CD8 (Wiest D.L. et al., 1993; Veillette A. et al., 1989) implies a more prominent role of the kinase in $CD4^{+}$ cell development. Consistent with the idea of qualitatively different signals defining the CD4/CD8 lineage fate, it seems likely that insufficient Lck recruitment is the reason for the generation of MHC class II-restricted $CD8^{+}$ T cells (Madrenas J. et al., 1997).

6.10. Partial TCR phosphorylation through elevated Fyn kinase activity provides an explanation for coreceptor reversal of the F2.5 T cell clone

Basson M.A. et al. (1998) proposed a model of lineage commitment in which CD4 versus CD8 lineage decision depends on the ratio of signals delivered by the TCR and Lck. The model ascribes CD8 differentiation to engagement of the TCR in the absence of overt Lck kinase activation, CD4 differentiation to modest pMHC-TCR interactions in the presence of high Lck activity and apoptosis induction to strong TCR and Lck signals. Accordingly, conversion of DP thymocytes into CD8 SP T cells may be the consequence of decreased Lck expression. In agreement with this hypothesis, immunoblotting of cell lysates from cocultures of $4^{-}/8^{hi}$ F2.5 T cell and antigen-loaded DC revealed reduced Lck expression. In contrast, Fyn, another Src family kinase expressed in T cells, was more readily detectable in $4^{-}/8^{hi}$ F2.5 T cells and, most importantly, could be further enhanced by

ECD/HER2-specific stimulation. Although Fyn may compensate for most of the functions of Lck (Groves T. et al., 1996), it has been shown that positive selection of CD4 SP thymocytes strictly depends on the expression of Lck (Zamoyska R. et al., 2003). An explanation for this finding is provided by the fact that only Lck leads to complete phosphorylation of the TCR/CD3 complex and downstream ZAP-70 kinase although both kinases bind to the ζ-chain of the CD3/TCR signal transduction complex (Mustelin T. and Tasken K., 2003). In the same immunoblot, dephosphorylation of ERK 1 and 2 was identified as a second event selectively induced by ECD/HER2-specific T cell activation. Although to date no relation between elevated levels of Fyn and dephosphorylation of ERK proteins has been mentioned in literature, both signals seem to act synergistically in the inhibition of CD4 T cell development since Bommhardt U. et al. (1999) showed that CD4, but not CD8 lineage commitment of thymocytes is highly affected by ERK kinase activity. Therefore it is likely that sustained ECD/HER2 antigen presentation leads to only partial TCR engagement and inhibition of downstream signaling, as suggested by the almost complete dephosphorylation of ERK proteins.

Evidence that reduced levels of Lck and overexpression of Fyn are not a rare phenomenon, but rather part of a common signaling pathway, is given by Huang J. et al. (2000). In a TCR transgenic mouse model, the authors observed selective activation of Fyn and partial T cell activation after MHC-restricted presentation of a low affinity antagonist peptide. This study is in agreement with several other studies showing that interactions with altered peptide ligands resulted in the transduction of distinct intracellular signals (Sloan-Lancaster J. et al., 1994; Madrenas J. et al., 1995; La Face D.M. et al., 1997; Chau L.A. et al., 1998; Hemmer B. et al., 1998). In every single case, the primary consequence of antagonist-induced signal transduction was altered ζ-chain phosphorylation and lack of ZAP-70 activity.

6.11. TCR signaling in ECD/HER2-stimulated $4^{-}/8^{hi}$ F2.5 T cells reveals a pattern of activation known to occur during positive selection in the thymus

From a virtually unlimited repertoire of receptor specificities created by random rearrangement of TCR genes only a small fraction is selected in the thymus on the basis of low avidity interactions with self MHC ligands (Goldrath A.W. and Bevan M.J., 1999). The signals for this positive selection of thymocytes are mainly provided by MHC-associated intrathymic self-peptides, as demonstrated by different authors (Sant'Angelo D.B. et al., 1997; Surh C.D. et al., 1997; Barton G.M. and Rudensky A.Y., 1999). In a TCR-transgenic mouse model, Williams O. et al. (1998) demonstrated that an anta-gonist peptide variant could inhibit negative selection of thymocytes induced by endogenously expressed peptide antigen. Most notably, this inhibition resulted in rescue and maturation of the thymocytes. Thus, their data indicate that positive selection is partially contributed to an antagonist-like engagement of the TCR. Direct evidence for the existence of antagonist peptides in a pool of naturally processed TCR determinants was provided by Santori F.R. et al. (2001). By using a sophisticated combination of bioinformatics and functional testing, these authors identified a self-peptide that both induces positive selection and acts as an antagonist of mature T cell proliferation. Accordingly, one

possible explanation for the initiation of signal transduction leading to coreceptor reversal on F2.5 T cells is that the putative epitope of the HER2 protein transduces antagonist-like signals.

The finding that positive and negative selection could be induced by different concentrations of the same peptide (Sebzda E. et al., 1994) indicates that the avidity of the pMHC-TCR interaction determines the outcome of T cell selection. Different avidities for the same peptide antigen (Lucas B. et al., 1999; Germain R.N. and Stefanova I., 1999), provide a possible explanation for the observation that from the established 12 clonal microcultures only the F2.5 T cell clone survived whereas the others underwent apoptosis even though all of them are derived from the same precursor cell as evidenced by constitutive expression of the TCR Vβ5.3 motif.

6.12. Absence of NIH3T3^{HER2}-specific IFN-γ release indicates reduced antigen-sensitivity of the $4^{-}/8^{hi}$ F2.5 T cell clone isoform

While $4^{-}/8^{hi}$ F2.5 T cells reliably responded to ECD/HER2, no HER2-specific IFN-γ production was observed for NIH3T3^{HER2} cell lysate as the antigen. Thus, it was concluded that small numbers of specific pMHC complexes failed to activate $4^{-}/8^{hi}$ F2.5 T cells. This behavior is in striking contrast to that of the two other F2.5 T cell clone subsets and that of the original F2 T cell line. Thus, $4^{-}/8^{hi}$ F2.5 T cells were analyzed for factors suppressing T cell activation. Indeed, several inhibitory mechanisms appeared to be operative. First, exceptionally high levels of the negative regulator CD5 were found on the surface of these cells. Secondly, as revealed by RT-PCR analysis, $4^{-}/8^{hi}$ F2.5 T cells expressed an alternatively spliced form of the CD8α molecule in which exon IV, coding for the transmembrane domain, was absent. This leads to a CD8α molecule being secreted and existing primarily as a homodimer (Norment A.M. et al.,1989). As was reported by Sewell A.K. et al. (1999) low concentrations of soluble CD8 can inhibit T cell activation and function *in vitro* and *in vivo*. In a model proposed by the authors, soluble CD8α competes with the membrane-bound form of the receptor for MHC class I binding, thereby leading to a blockage of receptor multimerization on the T cell surface.

6.13. KIR 2DL2 expression may be responsible for the induction of $4^{-}/8^{hi}$ F2.5 T cell proliferation by MaBa-LCL cells in the absence of HER2

Systematic analysis of proliferation and cytolytic activities of $4^{+}/8^{+}$ and $4^{-}/8^{hi}$ F2.5 T cells revealed a considerable degree of functional reactivity against autologous MaBa-LCL cells by the latter. Since KIR 2DL2 was present on a fraction of $4^{-}/8^{hi}$ F2.5 T cells, but was completely absent on $4^{+}/8^{+}$ F2.5 T cells, the observed reactivity was suggested to be mediated, at least in part, by this receptor. In NK cells, the KIR genotype determines self tolerance (Valiante N.M. et al., 1997) and allows these cells to detect "missing self", i.e., the loss of cell surface HLA class I expression arising from viral infection (Young N.T. et al., 2001). Several authors showed KIR expression on T cells to be mainly restricted to oligoclonally expanded CD8αβ^{+} T cells of activated or memory phenotype.

Additionally, it serves as a mechanism to inhibit lytic activity in response to chronic antigen stimulation (Mingari M.C. et al., 1996; Speiser D.E. et al., 1999; Huard B. and Karlsson L., 2000). The latter studies, however, were focused on the interaction of KIR receptors with HLA ligands actually expressed on the host cells. Since KIR 2DL2 selectively binds to group 2 HLA-C alleles, but donor MaBa only express the group 1 HLA-C alleles HLA-Cw4 and -Cw15, it must be assumed that KIR $2DL2^{+}$ $4^{-}/8^{hi}$ F2.5 T cells recognize KIR ligand mismatch on autologous cells. A lack of inhibitory control may therefore contribute to the induction of $4^{-}/8^{hi}$ F2.5 T cell proliferation by MaBa-LCL cells. This conclusion was further supported by results of a proliferation assay using a panel of allogeneic LCL cell lines with various HLA-C haplotypes as targets. In this study, it was found that $4^{-}/8^{hi}$ F2.5 T cells did not respond to LCL targets expressing the group 2 HLA-C alleles HLA-Cw3, -Cw5 and -Cw7. In addition, significant proliferation was detectable not only in response to MaBa-LCL cells but also in response to HLA-Cw4 homozygous LCL cells of cell line JB.

6.14. Enhanced sensitivity to a crossreactive EBV-epitope may explain the degenerate antigen specificity of $4^{-}/8^{hi}$ F2.5 T cells

Lack of KIR 2DL2-mediated inhibition alone is not sufficient for a complete explanation of this crossreactivity, since $4^{-}/8^{hi}$ F2.5 T cell proliferation was restricted to HLA-matched LCL cell lines, even though the T cells themselves were negative for group 1 HLA-C allels. This points to the conclusion that some degree of activating signals are additionally required. In this context, the detection of moderate CTL responses against DC pulsed with MaBa-LCL lysate revealed the involvement of a MHC-restricted pathway. Cytolysis occurred at a slightly higher effector-to-target ratio as compared to cultures with ECD/HER2 antigen. Moreover, in contrast to ECD/HER2 protein, presentation of MaBa-LCL cell-derived antigens was not sufficient to elicit a measurable IFN-γ response by $4^{-}/8^{hi}$ F2.5 T cells. Since Sykulev Y. et al. (1996) reported that the density of relevant pMHC complexes on target cells needed to produce a cytotoxic response is dramatically lower than the one required to stimulate cytokine production, it is likely that a lower affinity variant of the putative HER2 epitope was encoded by the EBV-transformed B cell line.

As it was demonstrated by various authors (Bhardwaj V. et al., 1993; Kersh G.J. and Allen P.M., 1996; Wucherpfennig K.W. and Strominger J.L., 1995), single T cell clones can be stimulated by diverse peptides and pMHC complexes. In this regard, Wucherpfennig K.W. (2004) noted that crossreactive peptides are characterized by a high degree of similarity at peptide residues creating the interface with the TCR, while differences occurred at sequence positions buried in pockets of the MHC binding site. An advanced model to describe the plasticity of T cell responses by Holler P.D. and Kranz D.M. (2004), known as conformer model, postulates the ability of the TCR to adopt multiple conformations with different peptide binding characteristics. Structural evidence for this model has been obtained from studies by Garcia K.C. et al. (1998) and Reiser J.B. et al. (2003). They found a high degree of flexibility of the CDR3 loops in the TCR binding site. According to the conformer model, crossreacting ligands can be true molecular mimics or structurally unrelated pMHC com-

plexes with an affinity for one single conformational state of the TCR. It is suggested by the authors that crossreactivity caused by either TCR flexibility or molecular mimicry is a frequent phenomenon or even a general feature of TCR recognition. Therefore, functional plasticity of T cell responses provides a logical explanation for the responsiveness of $4^{-}/8^{hi}$ F2.5 T cells to EBV-transformed B cells in the absence of the HER2 antigen.

Flexibility of TCR recognition seems, however, to be limited to peptide epitopes with low binding affinity (Holler P.D. et al., 2003). In this particular case, CD4 or CD8 coreceptor ligation seems to be essential for the initiation of T cell responses since it lowers the activation threshold by providing enhanced adhesion to target cells (Irvine D.J. et al., 2002; Holler P.D. and Kranz D.M., 2003). With regard to the efficiency of coreceptor function, CD8αβ is more potent in action than CD8αα. Thus, it appears that CD8β induction was beside partial KIR 2DL2 expression another prerequisite for $4^{-}/8^{hi}$ F2.5 T cell reactivity against MaBa-LCL cells.

Moreover, a role for CD27 in this activation event was suggested by the detection of significant amounts of the costimulatory receptor only on $4^{-}/8^{hi}$ F2.5 T cells. Since its corresponding membrane ligand, CD70, was detectable on LCL cell lines and not on immature DC, it was assumed that CD27-induced signal transduction occurred exclusively in cultures of $4^{-}/8^{hi}$ F2.5 T cells with LCL targets. Evidence that CD27-CD70 interaction reduces the threshold for T cell activation was provided by the observation that in mice constitutively expressing CD70 on B cells a spontaneous turnover of naive T cells into proliferating effector cells occurred even in the absence of deliberate immunization (Arens R. et al., 2001; Tesselaar K. et al., 2003). Interbreeding these mice with TCR transgenics pointed out that this process was driven by TCR signals, presumably induced by environmental antigens. Based on these findings, Hendriks J. et al. (2003) postulated that constitutive CD27 signaling improves the survival of T cells activated by low-level antigen expression. With regard to MaBa-LCL-induced $4^{-}/8^{hi}$ F2.5 T cell activation, transduction of CD27 costimulatory signals may promote low affinity crossreactive TCR interactions.

Taken together, the data suggest that inadequate antigen presentation by LCL cells led to the observed high expression of accessory molecules on $4^{-}/8^{hi}$ F2.5 T cells and that the resulting altered responsiveness contributed to the degeneration of antigen recognition by the $4^{-}/8^{hi}$ F2.5 T cell subset.

6.15. IL-12-mediated CD4 induction on $4^{-}/8^{hi}$ F2.5 T cells may indicate the preservation of Th cell responses under optimal antigen stimulation conditions

Addition of IL-12 to a $4^{-}/8^{hi}$ F2.5 T cell expansion culture induced expression of CD4, but also sensitized the cells to apoptosis. Therefore, it was concluded that IL-12-induced signaling in $4^{-}/8^{hi}$ F2.5 T cells interfered with signals that maintained the CD8 SP phenotype. The resulting signaling imbalance then promotes the activation of the death pathway (Evan G. and Littlewood T., 1998). This process, however, occurred late after IL-12 treatment, suggesting that extensive structural rearrangements may be involved. One suggestion is that T cells had to be primed by a crucial early signal,

which once delivered permitted the cells to react differentially to stimuli during the latter phase of the IL-12 response. Evidence for the existence of such an early and late IL-12 function in T cells is given by Abdi K. and Herrmann S.H. (1997).

Since IL-12 is mainly secreted by the DC during interaction with activated T cells (Snijders A. et al., 1998; Mackey M.F. et al., 1998), it was of particular interest to determine whether in a coculture of $4^{-}/8^{hi}$ F2.5 T cells and antigen-loaded DC IL-12 was produced in considerable amounts. As it was shown by ELISA, supernatants from cocultures of $4^{-}/8^{hi}$ F2.5 T cells and DC loaded with either ECD/HER2 antigen or NIH3T3^{HER2} lysate contained much higher amounts of IL-12 p40 than the IL-12-supplemented culture. Accordingly, it was suggested that $4^{+}/8^{lo}$ F2.5 T cells remained $CD4^{+}$ as long as ECD/HER2-loaded DC were used as APC and that stimulation with antigen-loaded LCL cells for cloning and expansion was the primary reason for the phenotypic changes leading to the development of the $4^{-}/8^{hi}$ F2.5 T cell subset.

6.16. Detection of DP T cells in human blood provide evidence for the natural occurrence of F2.5-like T cells

As a consequence of thymic selection, T cells in the periphery are tolerant to self-antigens and express either CD4 or CD8. However, as demonstrated in several studies not all autoreactive T cells are eliminated in the thymus (Ota K. et al., 1990; Moiola L. et al., 1993; Tandon N. et al., 1992). Furthermore, a small population of DP lymphocytes can be detected in the peripheral blood of healthy human individuals, particularly in elderly subjects (Blue M.L. et al., 1985; Zuckermann F.A., 1999; Ortolani C. et al., 1993). An increase of DP T lymphocyte frequencies was observed in individuals with neoplasia, infectious diseases or immune disorders (Suni M.A. et al., 2001; Sullivan Y.B. et al., 2001; Fujii Y. et al., 1999). A recent publication of Nascimbeni M. et al. (2004) clearly showed that circulating DP T cells in a chimpanzee were of an effector memory phenotype and sequestrated to the site of HCV infection. According to these findings it was evidenced that the generation of the DP F2.5 T cell phenotype was not due to an artifact of *in vitro* culture but rather reflected a naturally occurring maturation process. In this scenario, it is likely that the precursor cell of the F2.5 T cell clone was prematurely released from the thymus into the circulation. The course of its differentiation seemed to be largely influenced by the conditions under which the naturally processed ECD/HER2-derived peptide was presented. A suboptimal stimulation process by non-professional APC such as LCL cells may lead to only partial TCR signaling and to a degenerated antigen recognition, as evidenced in this study by the detection of enhanced Fyn kinase activity and crossreactivity to EBV-transformed B cell antigens presented in the context of HLA-Cw4. It remained, however, to be elucidated whether this is an exclusive property of the thymocyte-like F2.5 T cell clone or is a common feature of T cells subjected to suboptimal TCR stimulation. Finally, in agreement with recently published results (Germain R.N., 2002; Nishida T. et al., 2004), this study provides evidence that *in vitro* differentiation of F2.5 T cells was more influenced by the signaling end of the coreceptor than by the class of MHC that bound the antigenic peptide.

6.17. Conclusions

The results of the presented study provide proof of principle for successful isolation and expansion of HER2-reactive T cells from human peripheral blood using purified ECD/HER2 as the antigen stimulus. With regard to its application in adoptive immunotherapy, however, the success of this experimental approach is limited due to the difficulty to rapidly identify HER2-reactive T cells in the large peripheral T cell pool.

Besides earlier studies on vigorously responding T cells against self-antigens, such as myelin basic protein (Liblau R. et al., 1991; Mazza G. et al., 2002), MAGE (Toso J.F. et al., 1996; Coulie P.G. et al., 2002) or NY-ESO (Jäger E. et al., 1998; Valmori D. et al., 2000; Gnjatic S. et al., 2003), the establishment of a HER2-reactive T cell clone indicates that potentially autoreactive T cells are frequently present in the blood. These self-reactive T cells are still subject to stringent immunological control mechanisms (for review, see Anderton S. et al., 1999). This is clearly indicated by the fact that autoimmune diseases are relatively rarely found in population. One major aspect of this peripheral tolerance is the well-balanced ratio of Th1 and Th2 cells and their capacity to negatively crossregulate each other through their respective cytokines (Maggi E. et al., 1992; Romagnani S.,1995). Another aspect is the presence of $CD25^{+}$ regulatory T cells that actively suppress development of effector T cells (Danke N.A. et al., 2004). As a result of this strict control, it is generally considered that excessive activation of self-reactive T cells may only occur in certain pathological states, i.e. viral infections (Bach J.F., 2003; Salaman M.R., 2003), or may be induced *in vitro* by chronic exposure to self-antigens.

The main disadvantage of *in vitro* culture is the fact that only a minority of T cells is supported by the *in vitro* growth conditions (Thor Straten P. et al., 2000), although it is possible to generate TAA-reactive T cell clones even from healthy individuals. As a consequence, long-term T cell clones usually do not behave like memory T cells that participate in the *in vivo* immune response (Faure F. et al., 1998; Jenkins M.K. et al., 2001). Therefore, it is important to determine the optimal *in vitro* culture conditions needed for the successful generation of highly potent antitumor effector T cells. This is suggested in the presented study due to the fact that inappropriate stimulation of T cell clone F2.5 by LCL cells as APC have led to the dramatic changes in surface marker expression.

In particular, for T cell vaccine-based therapies, efficient protocols have to be established to permit rapid production of sufficient numbers of tumor-reactive T cells. Furthermore, it will be advisable to develop sophisticated T cell stimulation protocols for selecting and maintaining a phenotypically diverse T cell repertoire, when expanding these T cells under conditions of undisturbed homeostasis. This may be achieved by the following ingredients: total T cell preparations of the donor, tumor cell extract or a mixture of synthetic antigenic peptides as the antigen, autologous DC as professional APC and a balanced combination of growth factors and cytokines capable of exerting potent T cell growth and prosurvival signals. It may be possible in the future that the concept of *ex vivo*-induced polyclonal T cell activation will become the most applicable method of T cell-based immunotherapy for the treatment of a broad range of neoplasias with defined tumor antigens.

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