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Review Article

Induction/Engineering, Detection, Selection, and Expansion of Clinical-Grade Human Antigen-Specific CD8$^+$ Cytotoxic T Cell Clones for Adoptive Immunotherapy

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Adoptive transfer of effector antigen-specific immune cells is becoming a promising treatment option in allogeneic transplantation, infectious diseases, cancer, and autoimmune disorders. Within this context, the important role of CD8$^+$ cytotoxic T cells (CTLs) is objective of intensive studies directed to their in vivo and ex vivo induction, detection, selection, expansion, and therapeutic effectiveness. Additional questions that are being addressed by the scientific community are related to the establishment and maintenance of their longevity and memory state as well as to defining critical conditions underlying their transitions between discrete, but functionally different subtypes. In this article we review and comment latest approaches and techniques used for preparing large amounts of antigen-specific CTLs, suitable for clinical use.

1. Introduction

Adoptive transfer of antigen-specific cytotoxic CD8$^+$ T cells (CTLs) represents a promising approach especially in treating certain highly immunogenic tumors and viral infections. The production of clinical-grade effector T lymphocytes has evolved from a rather simple and clinically quite disappointing use of ex vivo lymphokine-activated killer cell (LAK) preparation, followed by the equally poorly effective and toxic systemic use of high concentrations of recombinant interleukin-2 (IL-2) to far more promising applications of in vitro expanded tumor-infiltrating lymphocytes (TILs) leading to and demanding technically complex cell bioengineering processes, requiring specific immune cell subtype isolation, clonal selection, genetic modification, as well as other extensive in vitro manipulations in order to obtain sufficient numbers of defined therapeutically effective cellular products. The understanding and considering of specific and complex mechanisms, characteristic of cell biology and physiology, are keys to successful ex vivo preparation and in vivo application of selected antigen-specific immune cells. Therefore we will first review the key mechanisms of CD8$^+$ T cell antigen recognition, their subsequent clonal activation, and effector function.

2. CD8$^+$ T Lymphocyte Antigen Recognition, Clonal Activation, Effector Function, and Memory Cell Pool Transition

Blood of normal individuals contains $5–12,5 \times 10^9$ T cells, among which are $2,5–8,4 \times 10^9$ CD4$^+$ and $1,5–4,5 \times 10^9$ CD8$^+$ T lymphocytes, resulting in a stable physiological CD4$^+$/CD8$^+$ cell ratio of 1,5. The size of the peripheral pool of naïve T cells is kept remarkably constant by poorly understood homeostatic mechanisms based on the perpetuum interplay between lymphogenesis, selective transition of newly emerged cells into a long-lived pool, the extent of lymphocyte survival in the periphery as well as their depletion following antigen-induced immune responses.

In order to differentiate into cytotoxic effectors or memory $CD8^+$ T lymphocytes, being able to traffic to nonlymphoid tissues, the naïve $CD8^+$ antigen-specific precursor T cells have to be activated within the lymphoid organs, especially in the immunogen-draining lymph nodes. Here the naïve cells first upregulate the expression of the chemokine receptor CCR5 which enables them to come into close contact with the sites of antigen presenting dendritic cell (DC)-antigen-specific $CD4^+$ helper T cell (T_h) interactions where the cognate attraction chemokines CCL3 and CCL4 are produced [1]. Finally, through the orchestrated cell-cell contacts, the optimal antigen-specific $CD8^+$ cytotoxic effector T cell clones (CTLs) are generated. The CTL responses to majority of antigens are T_h cell-dependent. Namely, T_h cells are able, through direct cell-cell interaction, to instruct and activate antigen-presenting cells (APCs) in such a way that they can then directly prime CTLs. This effect is referred to as the "licence to kill". Also, by paracrine secretion, T_h cells provide IL-2, needed for both their own clonal expansion as well as for starting and supporting the naïve $CD8^+$ T cell differentiation. Probably because of the fact that CTLs are so very much effective in delivering death signals to their targets, the naïve $CD8^+$ T cells require more costimulation in order to become fully armed cytotoxic effectors, when compared to $CD4^+$ T lymphocytes.

Beside a classical activation strictly by intracellular antigen presentation within the context of extremely polymorphic major histocompatibility complex (MHC) class I molecules, the so-called class I human leukocyte antigens (HLA), expressed on the surface of APCs, naïve $CD8^+$ T cells can also be stimulated via the so-called cross-presentation, resulting in cross-priming of precursor CTLs. This kind of CTL generation has first been evidenced and reported already in 1976, by Bevan [2]. It plays a role in the immune defence against many viruses (HPV, CMV, EBV, Influenza, Papilloma, ...) and several bacteria (Listeria, Salmonella, E. coli, ...) which do not infect APCs as well as against most types of tumors that can avoid immune surveillance through different mechanisms, for example, also by suppressing normal antigen processing pathways [3, 4]. Namely, not only certain APCs, primarily dendritic cells (DCs) and macrophages, but also, although less efficiently, B lymphocytes, neutrophils, liver sinusoidal endothelial cells, and bone marrow mesenchymal stromal cells, are able to take up and then process and present extracellular antigens through their class I MHC antigen-loading pathway [5–7]. The exact mechanism by which exogenous antigens reach the cytosol, where they enter the MHC class I antigen-processing and -loading machinery, is still unclear. However, professional APCs (DCs, macrophages) can engulf dead, infected and tumor cells or their debris by phagocytosis, receptor-mediated endocytosis, macropinocytosis, and pinocytosis and possibly also by seeping through gap junctions [8–10]. Endosomes are formed, containing the extracellular material, which fuse with lysosomes and the antigenic degradation starts. Once released in cytosol, the exogenous proteins are ubiquitylated to enter proteasome where they are cleaved in peptides. These are then translocated into the endoplasmatic reticulum (ER) by the heterodimeric transporter associated with antigen processing (TAP) and further degraded in smaller peptides, typically of 8 to 10 amino acids. The antigen loading set of nascent MHC class I–β_2-microglobulin molecular complex associated with tapasin, calreticulin, and ERp57 finally directs the peptide into the antigen-binding groove of the class I molecule. The antigenic peptide-loaded MHC class I molecules are then transported to the cell surface via the Golgi apparatus [11, 12]. Such primed cross-presenting APCs can thereby directly stimulate naïve $CD8^+$ T cells [13].

Basically, each effective T cell response to a given antigen depends on three complementary signals. The first one, defining its specificity, is provided by the interaction of the clonal T cell receptor (TCR), which in more than 90% is an immunoglobulin-like disulfide-linked $\alpha\beta$ transmembrane heterodimer, expressed on the responding antigen-specific $CD8^+$ or $CD4^+$ T cell and the antigenic peptide presented within the antigen-binding groove of a particular HLA class I (generally the intracellular antigens) or class II molecule (generally the extracellular antigens) present on the surface of a professional APC [14]. The genes coding for TCR α and β chains arise from rearranged germline-encoded gene segments. Genes encoding for the α chain are products of recombination of variable (V), junctional (J), and constant (C) gene segments, while those encoding for the β chain result from recombination of V, J, diversity (D), and C gene segments. The specific ligand-binding site of each TCR is composed out of two Vα and two Vβ encoded complementarity-determining regions or CDR loops (CDR1α, CDR1β, CDR2α, CDR2β) and two junctional CD3 loops (CD3α, CD3β). While the Vα and Vβ encoded CDR1 and CDR2 loops primarily interact with α helices surrounding the peptide-binding groove of a MHC molecule, the hyper variable CDR3 loop interacts with amino acid residues of the antigenic peptide, bound into the highly polymorphic peptides binding groove of the MHC molecule [15, 16]. The clonotypic TCR-MHC-antigenic peptide interaction is stabilised and enhanced by the interaction of T cell-specific $CD4^+$ monomers or homodimers with invariant parts of HLA class II molecules and in case of highly glycosylated $CD8^+$ $\alpha\beta$ heterodimers or $\alpha\alpha$ homodimers with the base of the constant α_2 domains of HLA class I molecules [17]. It has been shown that CD4 or CD8 binding to corresponding MHC molecules increases the sensitivity of T cells for antigen presentation by ~100-fold. Both, CD4, and CD8 bind Lck tyrosine kinase to their cytoplasmic tails and while interacting bring it close to the TCR [18]. When the reaction threshold between TCR, CD4/CD8, and antigenic peptide-MHC molecule complexes is reached, the activation signal is transmitted to the T cell nucleus. For this to be effective, a complete TCR complex is required. The CD3 molecular cluster, adjacent to the TCR and composed out of two ε, one δ and one γ transmembrane subunits, both stabilises TCR cell surface expression and is involved in activation signalling, as each of them has one immunoreceptor tyrosine-based activation motif (ITAM) present on their cytoplasmic structural region [19]. The TCR complex is also composed of a ζ chain homodimer having a very short extracellular domain and expressing 3 ITAMs

per long cytoplasmic tail of each ζ chain. Clustering of TCRs and their CD4/CD8 coreceptors on binding MHC-antigenic peptide complexes initiates signalling within the activated T cell through recruitment of CD4/CD8-associated Lck and activation of TCR receptor complex associated Fyn tyrosine kinases which in turn phosphorylate the CD3 ε, δ, and γ as well as ζ chain ITAMs [20]. Activation of these kinases is mediated by the tyrosine-specific phosphatase CD45, the so-called leukocyte common antigen. Subsequently the tyrosine kinase ZAP70 (zeta-chain-associated protein) binds to the phosphorylated ITAMs of each ζ chain and is then phosphorylated by the Lck. This enables the $\zeta\zeta$ homodimer to propagate, through complex signalling cascades, the activation signal onward to the T cell interior [21, 22].

The second signal is also crucial because if absent, regardless of the fact that the first antigen-specific signal is effectively accomplished, such primed T cells become anergic. Namely, in order to become fully operational and to undergo clonal expansion, the reacting T cells have to receive a proper costimulatory signal that is provided by interactions of T cell-specific CD28 molecules with their APC-expressed glycoprotein ligands B7.1 (CD80) and B7.2 (CD86) [23]. As already mentioned, the clonal expansion of $CD8^+$ T cells requires a stronger costimulatory signal than that of $CD4^+$ T lymphocytes. When naïve T cells become activated, they start to express a number of proteins able to sustain or modulate the costimulatory signal. One of them is the CD40 ligand (CD154) which binds to the CD40 molecule present on APCs. This interaction activates both T cells as well as APCs, which in turn start to express B7 costimulatory molecules [24]. The 4-1BB (CD137) costimulatory molecules, members of the tumor necrosis factor receptor (TNFR) family, are expressed on a minority of resting $CD8^+$ T cells and are transiently upregulated by all activated $CD8^+$ T lymphocytes. They bind their ligands (4-1BBL, CD137L) present on primed APCs (activated B cells, DCs, monocytes/macrophages) and thereupon promote $CD8^+$ T cell proliferation, differentiation, enhanced effector cytolitic capacity as well as the inhibition of apoptosis. Ligation of 4-1BB with 4-1BBL directly augments the cytotoxic function of activated human $CD8^+$ T lympocytes and additionally upregulates the expression of the activating natural killer (NK) cell receptor NKG2D on CTLs [25]. The latter effect is potentially important for the non-MHC-restricted elimination of infected or malignant cells, being able to downregulate MHC expression or shed various NKG2D ligands, for example, MHC-like molecules MIC-A and MIC-B. It has also been shown recently that while the CD28 co-stimulation pathway preferentially expands naïve $CD8^+$ T cells, the 4-1BB-induced signalling promotes proliferation of memory CTL populations [25]. Therefore, it seems that 4-1BB co-stimulation represents an important tool for ex vivo expansion of higly efficient antigen-specific memory CTLs for adoptive immune T cell therapies. One of the mechanisms that control the extent of proliferative responses of fully activated T cells depends on the ligation of CTLA-4 (CD152) and B7 costimulatory molecules. Namely, fully activated proliferating T cells start to express CTLA-4, which structurally closely resembles CD28. It binds approximately 20 times more avidly to B7 molecules than CD28, thereby delivering negative signals to activated T cells and limiting the autocrine production of T-cell growth factor IL-2. Additional inhibitory signals, regulating the extent of immune response (inhibition of cytokine expression, cell-cycle arrest, and/or apoptosis) also arise from the interactions of programmed cell death protein-1 (PD-1) expressed on T lymphocytes and its PD-L1 (B7-H1) and PD-L2 (B7-DC) binding molecules present on APCs [26]. Other important immune response regulators are: the transforming growth factor-β (TGF-β), which is prevalently produced by certain subsets of $CD4^+$ T cells (Th1, Th3, Tr1) and can inhibit growth as well as promote survival of T cells, and interleukin-10 (IL-10). IL-10 is produced by many different cell types, for example, B cells, macrophages, Th1, Th2, Th17, and Tr1 with the latter ones being regulatory cells that can be generated in the periphery from naïve $CD4^+$ T lymphocytes. Recent studies of regulatory T cells have clearly shown their importance in immunosuppressive modulation of cellular immune responses [27]. They are characterised by different phenotypes: $CD4^+CD25^+FoxP3^+$ (Treg), $CD4^+CD25^-IL10^+IFN\gamma^{low}IL4^-$ (Tr1), $CD4^+TGF\text{-}\beta^+$ (Th3), $CD8^+CD25^+$ that produce TGF-β and express CTLA-4, $CD8^+CD28^-$ (Ts) which induce the up-regulation of immunoglobulin-like transcripts ILT3 and ILT4 on DCs which in turn become tolerogenic; $CD8^+CD62L$(L-selectin)$^+CD122$(IL-2Rβ)$^+$, $CD8^+IL10^+$, and function through quite distinct mechanisms [28, 29].

The third signal is a polarizing one, determining the nature of cellular response, being either T helper type 1 (Th1), T helper type 2 (Th2), or T regulatory (Treg). It is mediated by interactions of both membrane-bound and prevalently soluble factors, such as interleukin-2 (IL-2), interleukin-7 (IL-7), interleukin-12 (IL-12), interleukin-15 (IL-15), type I interferons (IFN) or interleukin-10 (IL-10), produced by APCs as well as reactive T cells, and their respective cell surface receptors [30, 31].

The first division of fully activated T cells occurs after 48 hours and continues by 3-4 divisions per day, resulting in a 1000-fold expansion of the responding cell population within only few days. The initial Th1-dependent, autocrine IL-2-induced clonal CTL expansion is transient, and already within 3-4 days, the proliferating cells become activation-induced nonresponsive (AINR), and due to the lack of IL-2 production in the absence of additional exogenous IL-2 or appropriate $CD4^+$ T help, their proliferation ceases and their survival declines. However the TCR-mediated signalling remains operational, because following the recognition of MHC class I-antigenic peptide complexes expressed on target cells, the AINR CTLs produce IFN-γ and carry out their cytotoxic effector function. Antigen-specific lysis of target cells by armed CTLs can start already within 5 minutes and the killing process can endure over hours [32]. Cytotoxic T cells kill their targets by inducing their apoptosis or programmed cell death. Upon antigen-specific recognition and close interaction with target cells, CTLs, in a calcium-dependent way, release specialized lytic granules, which actually are modified lysosomes. These intracellular compartments contain different active cytotoxic proteins, which

are only effective when released form the granules [33]. One of them is perforin that, after polymerisation, forms irreversible pores in the membrane of attacked cells. The CTL lytic granules also contain granzymes, that is, at least three enzymes pertaining to the family of serine proteases and granulysin, being able to induce target cell apoptosis and possessing antimicrobial properties [33]. The surface mobilization of LAMP1 (CD107a), an integral cytolitic granule membrane protein, is a marker of degranulation after antigen stimulation and can be monitored by flow cytometry, for example in vaccine trials or in ex vivo expansion of CTLs for adoptive immunotherapy. As already mentioned, the granule-mediated killing is calcium dependent, but there is another apoptotic trigger that is operational even in the absence of Ca^{++}. Namely, in such conditions, $CD8^+$ effectors as well as some $CD4^+$ T cells, which constitutively lack lytic granules, are able to kill their targets. This involves binding of the Fas ligand (FasL, CD178), a member of the TNF molecular family, expressed on CTLs and some Th1 as well as Th2 cells, to Fas (CD95), present on antigen-specific target cells [34]. Ligation of Fas activates caspases, cysteine proteases, in target cells, that induce their apoptosis [35]. Most CTLs also produce and release IFN-γ, INF-γ, TNF-α, and TNF-β, thereby facilitating host defence against pathogens. They are able to directly inhibit viral replication, induce increased expression of MHC class I and other molecules involved in antigenic peptide presentation, as well as to activate macrophages [36]. Generally, according to the cytokine-producing profiles, two subtypes of cytotoxic $CD8^+$ T cells exist. The Tc1 cells have a cytokine secretion profile similar to Th1 lymphocytes while the Tc2 cells secrete cytokines similar to those produced by Th2 lymphocytes [37].

A complex and tightly regulated process involving multiple cell death-inducing mechanisms is also required for elimination of excessive effector T cells. Namely, it is well known that within few weeks following the pathogen clearance the vast majority (>90%) of effector T cells die. Beside negative signals blocking co-stimulation (CTLA-4) and the onset of increased sensitivity to inhibition by several cytokines, such as TGF-β, the Fas/FasL interaction represents a key mechanism triggering effector cell apoptosis, resulting in the regulation of peripheral immune response. However, a certain proportion of antigen-specific T cell effectors is salvaged and transformed into long-lived memory cells which respond more rapidly and effectively to pathogens that they have encountered previously, thereby reflecting the preexistence of the clonally expanded populations of antigen-specific T lymphocytes [38]. A strong correlation between memory cell production and the intensity of the primary cellular response exists. Interestingly, irrespective of the antigen quantity eliciting the immune response as well as the TCR-MHC-antigenic peptide affinities, the proportion of effector $CD8^+$ T cells surviving the postresponse phase in order to constitute the memory pool, is remarkably constant. It is always about 5% to 10% of the maximal cell numbers generated at the peak of the immune response. Such surviving $CD8^+$ T cells continue to express some of the surface markers characteristic of activated cells, for example CD44, but on the other hand they stop to do so with others, like the early activation marker CD69, a C-type lectin [39]. They also express more Bcl-2 protein, which promotes cell survival, and have substantially higher normal division rate (turnover) as well as apoptosis resistance in vivo than naïve T lymphocytes. Two basic types of memory $CD8^+$ T lymphocytes exist, the early or polarized effector (T_{EM}; $CD8^+CD27^-CD45RA^+CD62L^{lo}CCR7^-NKG2D^+$) and the resting or non-polarized central memory cells (T_{CM}; $CD8^+CD27^+CD45RA^-CD62L^{hi}CCR7^+NKG2D^+$) [40]. It has been shown that T_{CM} home to lymph nodes, while the T_{EM} cells preferentially migrate to gut and other lymphoid organs. The T_{EM} cells can rapidly mature into effector cells, containing perforin as well as secreting large amounts of IFN-γ, IL-4, IL-5, and low amounts of IL-2. They lack CCR7 chemokine receptors but express high levels of β_1 and β_2 integrins, strong, adhesion molecules, that bind also to extracellular matrix, suggesting that they can quickly enter inflamed tissue. On the other hand the T_{CM} cells are smaller than the T_{EM} ones and express CCR7, due to which they continuously re-circulate through T-cell zones of peripheral lymphoid tissues. As their fraction within the total antigen-specific T lymphocyte pool is rather small, they have a stem cell-like function in mounting strong recall responses whenever they reencounter the antigen. In fact they are very sensitive to antigen-specific TCR cross-linking and in response to it rapidly express CD40L. However they need more time to differentiate into effector CTLs than the T_{EM} cells and also secrete less cytokines following their restimulation. Namely, they produce only a certain amount of IL-2 and low quantities of IFN-γ, IL-4, and IL-5. It has also been shown in experimental animal models that the immunological memory can be adoptively transferred to naïve recipients and that the T_{CM} cells provide longer lasting immunity compared to the T_{EM} ones [41].

3. Some Important Factors Influencing Ex Vivo Preparation of Optimal Effector $CD8^+$ T Cells for Adoptive Immunotherapy

3.1. Detection and Selection of Starting T-Cell Populations. According to accumulating data it is very likely that precise detection and careful selective isolation of potentially most potent T cell subsets prior to their ex vivo expansion would enormously increase their adoptive transfer efficiency. Target T cell subset detection and selection can be performed under GMP conditions by specific monoclonal antibodies and specific antigenic peptide/selected HLA class I allele tetramer-based immuno(para)magnetic separation (Isolex 300i; Clini-MACS^R) or high-speed flow cytometry-based (FACS) cell-sorting as well as by the new non-aerosol-generating microfluidic cell sorting devices (Gigasort^{TM}), according to the expression of specific cell-surface markers informative of their biologic or clonal effector function. Beside the most common anti-CD3, anti-CD4 and anti-CD8, monoclonal antibodies can, for example, also be selected to recognize and select for CD62L (L-selectin), a C-type lectin leukocyte adhesion molecule; CCR7, a chemokine

receptor related to cell homing, and various isoforms of the protein tyrosine phosphatase CD45, that is, CD45RA present on naïve/nonactivated and CD45RO on activated T lymphocytes. It seems more and more so, that the selection of T_{CM} ($CD62^{hi}CCR7^{+}$) lymphocytes could provide a starting source of adoptively transferable immune cells with improved in vivo persistence and therapeutic efficacy [42].

In humans, peripheral blood, tumor tissue, and draining lymph nodes have until now been sources of T cells for adoptive transfer of antigen-specific immunity. Recently it has become clear that bone marrow is a major reservoir of self-reactive T cells with a potential for their adoptive transfer premanipulation. For example, it has been shown that the bone marrow of breast cancer patients contains $CD8^{+}$ T cell clones which specifically recognize peptides representing the immunogenic part of the MUC1 and HER2/neu tumor antigen aminoacid sequences, and also that, in pancreatic cancer and myeloma patients, this haematopoietic compartment is enriched for TAA-reactive CTLs [43]. Therefore it remains to be determined whether or not the improved antitumor effects can be observed after adoptive transfer of these specific $CD8^{+}$ T cell clones.

3.2. $CD4^{+}$ T-Cell Help. Due to the fact that IL-2 is needed for effective in vitro expansion of antigen-specific $CD8^{+}$ CTLs, thereby providing conditions that at least partly substitute the $CD4^{+}$ T-cell help, it is quite obvious that a concomitant presence of helper T cells is important for the ex vivo production of high numbers of cytotoxic effector T cells. This statement is even more convincing as $CD4^{+}$ T cell help is required for the onset and/or maintenance of the $CD8^{+}$ T cell memory, closely related to increased in vivo survival of CTLs as well as for enhancing the cellular immunity against tumors lacking MHC class II expression. Namely, adoptively transferred $CD4^{+}$ T lymphocytes are able to augment antitumor and antiviral immunity by several mechanisms involving the enhancement of effector $CD8^{+}$ T cell function and survival, secretion of essential cytokines, such as IL-2 and IL-21 as well as expression of CD40L [43]. It has been clearly demonstrated that the persistence of adoptively transferred $CD8^{+}$ effector T cells increased either with concomitant application of IL-2 or $CD4^{+}$ T lymphocytes [44]. Finally, recent studies in myeloma patients have additionally shown that the adoptive transfer of mixed populations containing antigen-specific $CD8^{+}$ and $CD4^{+}$ T effector T cells promoted the onset of immunity with establishing a robust central memory component [45].

3.3. Cytokines. Beside IL-2, additional cytokines have been extensively tested and some of them also used for ex vivo preparation and in vivo transfer of adoptive immune cells. The common gamma chain (γ_c; CD132) is a structural component of IL-2, IL-4, IL-7, IL-9, and IL-15 binding receptors. Cytokines that signal through γ_c are absolutely indispensable for T cell development in humans [43]. Besides sharing the γ_c, IL-2 and IL-15 receptors also use a common β chain (CD122) and induce T-cell growth-promoting stimuli through Jak1 and Jak3 signalling pathways. The unique IL-15α receptor is responsible for inhibition of activation-induced cell death. Additionally IL-15 prevents Fas-induced apoptosis and its presence overcomes tolerance of tumor-specific T cells. Therefore a concomitant use of IL-2 and IL-15 seems to be of benefit in adoptive immune cell preparation and transfer [46]. However, it has been recently shown that IL-2 might also exert negative effects by depleting memory T cells and increasing the number of tumor-protecting regulatory T cells (Tregs) [47]. By contrast, the two homeostatic cytokines, IL-7 and IL-15 seem to increase the persistence of $CD8^{+}$ memory T lymphocytes and might, therefore, decrease the ratio between Tregs and effector T cells [48].

3.4. Costimulatory Molecules and Their Counterreceptors. The surface expression of costimulatory molecules, including those pertaining to the B7 family (CD80, CD86, ICOS ligand, the inducible costimulator ligand) and members of the TNF family (4-1BB ligand, OX40 ligand), is induced in APCs (macrophages, B cells, DCs) following their activation. Except for CD28 which is constitutively expressed on resting T cells, their counterreceptors (ICOS, 4-1BB, OX40) appear exclusively on activated T lymphocytes requiring several days to achieve peak densities. Their expression greatly diminishes when effector $CD8^{+}$ T lymphocytes differentiate into memory cells. The delayed appearance of these costimulatory receptors on activated $CD8^{+}$ T cells supports the hypotheses that they are involved in sustaining the ongoing cellular immune response as well as supporting the survival of the newly induced CTLs [49]. Yet another molecule, the CD83, a member of the immunoglobulin superfamily, which is restrictively expressed on activated professional APCs, most notably mature DCs, has recently been suggested to have important role in priming naïve $CD8^{+}$ T lymphocytes by driving their antigen-specific expansion and supporting their function as well as long-term survival (>6 months). Its corresponding receptors (CD83L) appear on antigen-specific $CD8^{+}$ T cells only following TCR and CD28 signalling [50]. Their engagement enhances proliferation of both, the newly primed naïve as well as memory $CD8^{+}$ T cells and inhibits effector cell apoptosis. Therefore CD83, together with other costimulatory molecules, appears to be an important candidate for standardized highly-efficient antigen-specific artificial antigen-presenting cell (aAPC) preparation which is progressively substituting the classical use of autologous professional APCs (DCs, monocytes/macrophages, B cells).

3.5. Telomere Length. Telomeres, composed of TTAGGG repeats at chromosomal ends, are involved in cell proliferation and regulation of senescence. They also function as tumor suppressors, protecting chromosomes from degradation, fusion, and recombination. The telomeric repeats are synthesized onto chromosomes by telomerase which is a ribonucleoprotein enzyme. In all human cells, except germ and stem cells as well as some activated lymphocytes, telomeres lose a portion of their noncoding DNA repeats with each division. This shortening of telomeric DNA represents one of the mechanisms leading to cell senescence,

so the telomere issue should be considered in preparing immune cells for adoptive transfer. It has been shown that in human $CD8^+$ T lymphocytes telomeres are shorter in $CD8^+CD27^-CD45RA^+T_{EM}$ and $CD8^+CD27^+CD45RA^-T_{CM}$ memory cell subsets as compared to the yet unprimed $CD8^+CD27^+CD45RA^+$ naïve T cell population [51]. Therefore it seems probable that for optimal adoptive T cell therapies the preservation of telomere length and replicative life span capacity of memory cells is crucial for a long-term immune protection, even more so, as it has recently been shown that this correlates with the engraftment efficiency and increased antitumor activity of adoptively transferred T cells in melanoma patients [52]. In fact, TIL clonotypes that were effective and persisted in vivo had mean telomere length of 6.2 kb in comparison to 4.5 kb within those, that were not persisting. It is well known that in humans, stem cells and lymphocytes, are able to induce telomerase activity [53]. In effector T cells, costimulatory signals are required for this induction [54]. The CD28 co-stimulation maintains telomere length in T cells, therefore the in vitro cell culture conditions that promote costimulatory activity might ameliorate engraftment and in vivo persistence of adoptively transferred T cells. The application of IL-7 and IL-15 during the cell culture and/or after cell infusion could be beneficial as it has been shown that IL-15 induces sustained level of telomerase activity, thereby minimizing telomere loss in $CD8^+$ memory T cells, even after substantial number of their divisions [55, 56].

4. In Vitro Tests for Detection and Functional Evaluation of Antigen-Specific $CD8^+$ Effector Cells

4.1. Cell-Mediated Lymphocytotoxicity (CML) and Limiting Dilution Analysis (LDA). Standard ^{51}Cr-release cell-mediated lymphocytotoxicity (CML) assay is a classical test in which the specificity and cytotoxic potential of CTLs, previously generated under defined cell culture conditions, are assessed. By establishing microcultures, containing different effector to $Na^{51}CrO_4$-containing specific target cell ratios (E : T) and their subsequent 4 hour incubation at 37°C, the amount of ^{51}Cr, specifically-released from destroyed targets is finally detected by a γ-counter [57].

With a use of limiting dilution assay (LDA), antigen-specific CTL precursor (CTLp) frequencies can be defined in a population of PBMCs by performing assay evaluation based on Poisson distribution statistical analysis. At least 24 replicate microcultures for each of seven different, normally twofold dilutions of responding PBMC are set up and cultured for a defined period of time in the presence of a constant number of stimulator cells and rhIL-2. Constant numbers of specific, $Na^{51}CrO_4$-containing specific target cells are then added to each microculture. After additional 4 hour incubation at 37°C, the supernatants are harvested and the CTL activity assessed by measuring the amount of ^{51}Cr, specifically-released from destroyed targets in each microcultures. The CTLp frequency can be determined both, statistically by applying minimum χ^2 and/or maximum likelihood analysis and graphically, by plotting on the log scale the percentage of negative microcultures (ordinate) against the numbers of responding cells per each dilution (normal scale, abscissa); the plot should result in a straight line [58].

4.2. Enzyme-Linked Immunospot (ELISPOT) and Multiparameter Flow Cytometry-Based Intracellular Cytokine Staining Assays (ICS). The ELISPOT assay has been developed from a traditional ELISA technique and is used for detecting and evaluating local concentrations of selected cytokines produced by individual antigen-activated T cells, for example IFN-γ secreted by activated $CD8^+$ effector T cells [59, 60]. A capture, cytokine-specific antibody is fixed on a pre-coated polyvinylidine fluoride (PVDF) plate and blocked with a serum. Subsequently the cells are added together with an appropriate stimulant and the plates incubated to allow the antibodies to bind the specific cytokine. After washing, a biotinylated antibody is added to enable detection of the captured analyte, followed by the individual, cytokine producing cell visualization step, requiring the addition of avidin-HRP (HRP—Horse Radish Peroxidase) or avidin-ALP (ALP—Alkaline Phosphatase) conjugate and a coloured precipitating substrate. The ELISPOT assay is very sensitive and allows quantitative as well as qualitative assessment of the cytokine production.

Upon recognition of their cognate antigen, the subsequently fully activated T cell clones start to produce a range of different cytokines that can be detected within a single cell by ICS assay. In order for this to be possible, the secretory pathway of selected cytokines must be blocked by Brefeldin A, a metabolite of fungus *Eupenicillium brefeldianum* that specifically blocks protein transport from the ER to the Golgi apparatus, thereby causing their intracellular accumulation, which in turn can be detected by appropriate antibody staining and flow cytometry [60, 61].

4.3. HLA Class I Multimer-Based Assays. The first reagents of this kind were HLA class I tetramers that were at first used for in vitro quantitative determination of $CD8^+$ T cell clones according to the affinity and binding specificity of their TCRs, to a defined tetrameric HLA class I/antigenic peptide complex. First, soluble α chains of target HLA class I molecules, bearing specific amino acid sequence at their carboxyl terminus, recognised by the enzyme BirA, are synthesised by *E. coli*. After the addition of beta-2 microglobulin (β_2m) and the chosen antigenic peptide, the soluble HLA molecules are correctly folded and in the presence of BirA, one biotin per HLA molecule is attached. When fluorochrome-tagged streptavidin, a molecule with four biotin-binding sites is added, homogenous tetrameric HLA class molecule/antigenic peptide complexes are formed possessing high affinity for binding cognate TCRs [58]. Such tetramers are then used, together with other fluorescently labelled monoclonal antibodies specifically recognising defined T cell-specific surface molecules, for precise quantitative characterisation of target T cell clones. As the tetramer-bound T cells that can be obtained directly

from fresh peripheral blood and tumor tissue or previously ex vivo activated PBMCs, remain viable, they can be selected by cell sorting, for further characterization and/or expansion. Recently improved reagents, that is, pentameric HLA class I allele/antigenic peptide complexes were developed and are commercially accessible (Pro5 MHC Class I Pentamers, Proimmune). Due to their planar configuration, all five HLA-peptide complexes, assembled through a coiled-coil domain, are available for binding to complementary TCRs, while in tetramers, due to the tetrahedral spatial organization, only three out of four MHC-peptide complexes are available for TCR interaction.

5. Endogenous and Ex Vivo Induced Antigen-Specific CD8$^+$ T Cells, Expanded In Vitro for Subsequent Adoptive Immunotherapy

During recent decades the procedures for ex vivo selection, isolation and propagation of antigen-specific effector cells pre-primed in vivo or induced in vitro have been successfully developed, allowing preparation of clinical-grade CTL effectors, directly targeting various tumor associated antigens (TAA) as well as different viruses, for example cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus (ADV), herpesvirus (HPV), human immunodeficiency virus (HIV) and influenza-A virus.

5.1. Tumor-Infiltrating Lymphocytes (TILs). Autologous TILs can be obtained from excised solid tumors, tumor-draining lymph nodes and even from peripheral blood of cancer patients, sometimes following the precedent vaccination with TAA in order to increase the frequency of circulating anti-tumor precursor immune cells. Unfortunately only 30–40% of all tumor biopsies have been reported to yield satisfactory T cell populations [46]. For example, $\geq 1\,cm^3$ pieces of freshly resected metastatic melanomas were required for obtaining sufficient starting numbers of TILs [62]. The relatively small amounts of TILs that can be isolated from different types of cancer tissue biopsies, represent a polyclonal mixture of different immune cell types: CD4$^+$ and CD8$^+$ T lymphocytes, NK cells, DCs, monocytes/macrophages and B cells. By using appropriate cell culture conditions, favouring T lymphocyte proliferation, a massive expansion of pre-selected TILs, while retaining their antigen specificity, is technically feasible and can result, within several weeks, in a final dose of $>10^{10}$ clinically applicable cells [42]. The re-infused CTLs can make up to 37% of the total transferred TIL population. Immediately after cell isolation from fresh biopsies, multiple cell cultures are established, separately grown and then assayed in vitro for specific T cell tumor recognition, for example by using ELISPOT tests, detecting cytokine-secreting activated effector cells. Only those single cultures that exert high anti-tumor T cell reactivity are then selected for massive expansion.

The use of defined serum-free media (X-VIVO or AIM-V), usually high doses of recombinant human IL-2 (rhIL-2)—up to 6000 U/ml, γ-irradiated allogeneic peripheral blood mononuclear cells (PBMCs) or clinical-grade EBV-transformed B lymphoblastoid cell lines (LCLs) as feeder cells, enable the creation of optimal T cell expanding culture conditions that can be standardized and applied within the constraints of the good manufacturing practice (GMP) [42]. Under such, so-called rapid expansion protocol (REP) conditions, developed by Riddell et al., TILs can be polyclonally stimulated by a combination of anti-CD3 (OKT3) and anti-CD28 antibodies, with the first ones triggering the initial T cell activation signal through cross-linking of CD3-ζ elements of the TCR complex and the second ones providing proper costimulatory signals following their interaction with CD28 molecules [63]. The use of PBMC/LCL feeders can be substituted by various kinds of antigen-non-specific artificial APCs (aAPCs), equipped with number of surface bound T cell activating and costimulatory molecules: anti-CD3 antibodies; anti-CD28 antibodies and other costimulatory signal and prolonged TIL lifespan providing molecules, such as 4-1BB, CD40 and adhesion molecule co-stimulator OX40 (CD134), a member of the TNF family. Such aAPCs can also be prepared in a standardized way under GMP conditions. For this purpose magnetic beads, on which the activating and costimulatory molecules can be bound chemically or alternatively the MHC class I-negative leukaemia cell line K562, transfected with 4-1BB and CD32 (FcγRII), a low affinity Fc receptor (K32 cells) which represents the anchor for anti-CD3 and CD28-specific antibodies, have successfully been used [64, 65]. However, these non-specific aAPCs preferentially expand CD4$^+$ in comparison to CD8$^+$ T cells, which, during the process, additionally lose a significant proportion of their antigen-specific CTL activity unless a proper in vitro antigen-specific re-stimulation is provided. Therefore new approaches in developing aAPCs will be needed for efficient ex vivo generation of large amounts of antigen-specific CD8$^+$ TILs. Recently it has been reported that aAPCs, generated by using biotinylated activating (anti-CD3), costimulatory (anti-CD28) and adhesion (anti-LFA-1; CD11a) monoclonal antibodies, pre-clustered in microdomains, established on liposome scaffolds by pre-inserted neutravidin rafts, could efficiently induce expansion of endogenous antigen-specific CTLs [66]. However, prolonged stimulation/expansion of CD8$^+$ TILs causes their terminal differentiation, exhaustion and shortens their life span in vivo.

As IL-2 is crucial for effective TIL expansion, the monitoring by flow cytometry of two important factors involved in IL-2-mediated T cell activation, namely CD27 costimulatory molecule, a member of the TNFR family, expressed on non-activated T cells, NK cells as well as on some B cells, and its CD70 molecular ligand that is abundantly present on the surface of activated T cells, B cells and macrophages, has revealed important insights regarding their molecular dynamics and function. Namely both, in vivo and in vitro application of high IL-2 concentrations up-regulates the CD70 and at the same time blocks the expression of CD27 molecules on the surface of CD8$^+$ T cells. The withdrawal of IL-2 from activated CD8$^+$ T lymphocytes, previously

maintained in the presence of the cytokine, results in a reversal of the expression of these two molecular markers. The proliferation of T cells, stimulated with IL-2, primarily occurs in a subset of $CD8^+CD70^+$ T cells that have up-regulated IL-2 receptor expression, but not in $CD8^+CD70^-$ T lymphocytes. Furthermore, the analysis of TIL samples that were administered to melanoma patients showed that the size of the $CD8^+CD27^+$ T cell pool within each particular bulk TIL preparation was highly associated with the induced antitumor activity [67].

5.2. Adoptive Transfer of Endogenous Virus-Specific CTLs. Viral infections represent a major cause of morbidity and mortality during the first, highly critical 100 days of the post-transplantation immune recovery of myeloablatively pre-conditioned patients, having received haematopoietic stem cell (HSC) transplantation with a concomitant immuno-suppressive medication. Therefore, adaptive transfer of HSC donor-derived antiviral $CD8^+$ T cells represents effective treatment resulting in reconstituted antiviral immunity in such heavily immunocompromised recipients. The simplest approach is to use unmanipulated donor lymphocyte infusions (DLIs), containing high frequencies of virus specific effector T cells recognizing and eliminating common latent viruses, such as CMV and EBV. Otherwise, DLIs are quite routinely administered to generate graft versus leukaemia effect (GvL) in those HSC transplanted patients that have relapsed after they had been tapered off immunosuppressive drugs, with no evidence of severe graft versus host disease (GvHD). Detection, isolation and ex vivo expansion of virus-specific CTLs originating from allogeneic, MHC haploidentical or identical (siblings) CMV and/or EBV seropositive individuals have also been successfully carried out and the resulting cell preparations used for treating viremia as well as post transplant lymphoproliferative disease (PTLD) and virally caused malignancies in immunocompromised patients. Anyhow, DLIs and other allo- or semiallogeneic cellular products contain a high frequency of alloreactive cells that could give rise to GvHD. Therefore, in order to reduce this risk, donor T cells can be transduced with suicide genes which are triggered if the recipient develops GvHD. In this way, such cell preparations can be administered already early post transplant, reconstituting virus-specific immunity and at the same time allowing control over GvHD. The most commonly used and clinically tested is the herpes simplex virus thymidine kinase (HSV-tk) that renders transduced T cells sensitive to the antiviral drug ganciclovir [68]. Unfortunately this enzyme is a foreign antigen to recipient and therefore the transduced cells can be quickly eliminated by CTLs. Therefore new non-immunogenic suicide genes based on the Fas- and Caspase 9-FK-binding domain chimeras have been prepared that are able to induce target T-cell apoptosis via dimerization process, activated by a non-toxic chemical substance [69]. Alternatively, selective ex vivo depletion of alloreactive T cells has also been used to minimize the perils of generating GvHD after the adoptive transfer and proved to be successful, as small numbers of residual MHC haploidentical donor cells accelerated anti-viral immune responsiveness in the recipient [68].

5.3. Cytomegalovirus (CMV). CMV is a lytic virus, latently present in approximately 70% of healthy individuals. Cell-mediated immunity is considered to be crucial in controlling the infection with both $CD4^+$ and $CD8^+$ T cells importantly involved in antiviral immune protection. This has been proven in several cases where CMV-specific CTLs were infused into patients 30–40 days post HSC transplantation and were found effective but persisted only in those recipients whose $CD4^+$ T-cell-mediated CMV-specific immune responses were recovered [70]. Also, when both CMV-specific $CD4^+$ and $CD8^+$ T cells were transferred into patients, long-term persisting immunity was achieved [71]. A majority of anti-CMV CTL therapies described till recently, required long periods of ex vivo $CD8^+$ T-cell activation and expansion in the presence of IL-2, and were depending on specialized GMP conditions. Recently, tetramer MHC-based selection of CMV pp65 peptide-specific T cells directly from peripheral blood, their subsequent adoptive transfer, resulting in their expansion by several logs in vivo and a successful clearance of infection in the majority of recipients, has been reported [72]. By using another approach, DCs were prepared from adherent fraction of fresh PBMCs isolated from CMV seropositive HSC donors and used to stimulate autologous T lymphocytes for 21 days in the presence of IL-2. Direct positive immunomagnetic selection of same donor IFN-γ-secreting T cells from leukapheresis units, following a short pre-incubation with the recombinant CMV-specific pp65 antigen, was also carried out. Both alternatively generated CMV-reactive T cells were successful in accelerating reconstitution of antigen-specific immunity in patients following HSC transplantation [73]. Efficient CMV-reactive $CD8^+$ and $CD4^+$ T cells can also be prepared by using selected single human leukocyte antigen (HLA) class I or class II allele-specific aAPCs. Mouse 3T3 fibroblasts were therefore sequentially transduced with retroviral vectors to express human costimulatory (B.71) and adhesion (ICAM-1, LFA-3) molecules, β_2-microglobulin and α chains of different HLA class I alleles (A*0201, A*0301, A*2402, and B*0801). Such aAPCs were then transduced to co-express a full length antigenic CMVpp65 protein and were able to elicit strong HLA-restricted anti-CMV CTL responses, not only against dominant but also subdominant pp65 antigenic epitopes, as clearly shown by the particular peptide/HLA class I allele tetramer binding assays in vitro [74].

5.4. Epstein-Barr Virus (EBV). EBV is a γ-herpesvirus, latently infecting more than 95% of the world's population. There are at least four known types of viral latency, characterized by expression of numerous different EBV-specific antigens with prevalently low and rarely high immunogenicity that are related to different diseases [68]. While the type 1 is common to Burkitt lymphoma and gastric carcinoma, the type 2 is observed in Hodgkin lymphoma, nasopharyngeal carcinoma, NK and T cell lymphomas and other mucosal carcinomas, with both types obviously being able to evade immune surveillance due to a combination of low immunogenicity of expressed EBV-related proteins and the presence of anti-inflammatory cytokines TGF-β and

IL-10, produced by reactive T lymphocytes and the Reed-Sternberg cells [75]. On the other hand the type 3 latency, involving genes coding for all EBV-associated proteins, also the most immunodominant nuclear antigens, EBNA3A, -3B and -3C, being appropriate targets for immunotherapy, is responsible for causing lymphoproliferative disorders, but only in severely immunocompromised patients having received allogeneic HSC or organ transplants (PTLD), being congenitally immunodeficient or HIV-infected.

The EBV-infected B cells in PTLD express the same phenotype and virus-specific antigens as experimentally used EBV-transformed lymphoblastoid cell-lines (LCLs), prepared in vitro by infecting peripheral blood B lymphocytes with a laboratory strain of EBV. As EBV-transformed LCLs can be readily generated from any donor, they are used as APCs for preparing clinical grade polyclonal anti-EBV $CD4^+$ and $CD8^+$ effector T lymphocytes, recognizing multiple latent and lytic viral antigens. In this way high numbers, that is, $>10^7/m^2$ of EBV-reactive CTLs can be produced, which are sufficient for repeated cycles of their adoptive transfer [76, 77]. Such adoptively transferred CTL effectors survive up to eight years in the recipient and can expand up to 2–4 logs after infusion [68].

Unfortunately nasopharyngeal cancer (NPC) and Hodgkin disease (HD) do not express immunodominant EBV antigens, instead the subdominant EBNA1 is characteristic of NPC and the latent membrane proteins 1 and 2 (LMP1, LMP2) for HD and 50% of NPCs [78]. Clinical trials in which CTLs specific for these antigens were used for treatment, have only been partially successful. In order to improve their potency, the subdominant EBV antigen-specific CTLs were genetically modified ex vivo to make them TGF-β resistant [79].

5.5. Adenovirus (ADV). Adenovirus is a non-enveloped lytic DNA virus with 51 serotypes, forming six distinct groups (A-F). The adenovirus infection is very frequent in paediatric HSC transplantation. A group of patients with ADV infection were successfully treated with a combination of virus-specific polyclonal $CD4^+$ and $CD8^+$ T lymphocytes obtained from HSC donors, shortly stimulated in vitro with viral antigen, actually a lysate, prepared from ADV Spc-infected human embryonic lung fibroblasts (HELF), subsequently immunomagnetically selected according to their IFN-γ production and then expanded in the presence of IL-2 and feeder cells [68, 80].

5.6. Multivirus-Reactive T cells. Such pluripotent antiviral T lymphocytes, able to eradicate CMV, EBV and adenovirus infected cells, can be generated and expanded in vitro by using autologous or donor-derived PBMCs transduced with a recombinant adenoviral vector, encoding for the highly immunogenic CMV antigen pp65 as stimulators. Subsequently, EBV-transformed LCLs, transduced with the same vector are added as APCs, thereby additionally activating EBV-specific T cells as well as supporting previously activated anti-CMV and adenovirus reactive T lymphocytes [81]. The application of such ex vivo induced and expanded polyclonal CTLs resulted in relevant, clinically detected clearance of combined infection with all three viruses. Alternatively, DCs can also be used as efficient inducers of adoptively transferable anti-multiviral T cell effectors.

6. In Vitro Induction/Engineering and Expansion of Clinical-Grade Antigen-Specific CTLs

Besides the direct use of endogenously developed CTL responders that can optionally be additionally amplified before their expansion ex vivo, de novo engineered antigen-specific HLA class I-restricted immune reactions can also be induced in vitro by using responding PBMC population and various types of stimulating APCs, either the natural professional or artificially created ones.

6.1. Professional APCs. Professional antigen presenting cells, macrophages, B cells and the most potent DCs possess all the necessary machinery for antigen uptake, processing and MHC-restricted presentation as well as costimulatory signalization and cytokine production. The most frequently exploited for antigen-specific CTL generation are DCs which can be prepared in vitro either from bone-marrow-derived $CD34^+$ cells or, prevalently from easily accessible peripheral blood $CD14^+$ monocytes [82]. Besides the optimal in vitro preparation of DCs, the most effective antigen loading method is the next crucial step in providing strong and specific (re)activation of T cells. Of special interest is the presentation of TAAs. As some of them, especially those specific for melanoma, are well defined, synthetic antigenic peptides with high binding affinity to specific HLA class I alleles can be used. Alternatively native peptides eluted from HLA alleles, expressed on the surface of tumor cells can be applied. Also whole proteins or tumor cell preparations (apoptotic cells, cell lysates) can be delivered to immature DCs which have a strong capacity to uptake, process and present their peptide fragments within the context of both, HLA class I and class II molecules. Following their maturation, such antigen loaded DCs are strong inducers of specific $CD4^+$ and $CD8^+$ T cell immune responses. Whenever appropriate amounts of tumor cells are available, hybrid cells following their chemical or electrofusion with DCs can be produced, therefore combining the whole spectrum of TAA and stimulatory properties of professional APCs. We have recently shown that the amount of fused late endocytic compartments correlates with the potency of in vitro CTL responses generated with electrofused tumor cell-DC hybridomas [83]. Also tumor-derived DNA and especially RNA can be transferred into DCs, thereby providing the genetic information for complete TAA spectrum to be produced, processed and efficiently presented to T cells. Several laboratories, including ours proved the effectiveness of native and/or amplified tumor mRNA transfection in generation of potent antitumor CTLs [57, 82]. The mRNA transfection approach offers a practical solution, obviating the need for extensive amounts of tumor tissue which can be a limiting factor for antigen-specific DC preparation.

It is also still not clear to what extent DCs depend on the concomitant presence of $CD4^+$ T cells in the induction of CTLs. Nevertheless the promoting effect on $CD8^+$ T lymphocyte differentiation has been clearly shown for a number of cytokines, for example IL-2, IL-12, IFN-γ and IL-15. Manjutah et al. have shown that a certain threshold concentration of IL-2 is needed for the CTL effector differentiation from their precursors, while IL-15 alone or the subtreshold concentrations of IL-2 can only promote $CD8^+$ T-cell proliferation and their IFN-γ production [84].

6.2. Artificial Antigen-Specific APCs (aAPCs). Besides the already mentioned use of standardised "off the shelf" antigen-non-specific aAPCS, HLA-allele/antigenic peptide complex transduced surrogates of natural professional APCs can also be engineered and subsequently used in vitro to induce tailored antigen-specific cytotoxic immune responses of $CD8^+$ T lymphocytes that can be adoptively transferred into patients. Two basic approaches for preparing MHC-class I-antigenic peptide or MHC-class II-antigenic peptide specific aAPCs, using either cell lines or acellular systems, can be used. HLA class I antigen-specific aAPCs, able to efficiently stimulate $CD8^+$ cells can for example be produced by using the following:

(a) *drosophila melanogaster* cells which are unable to load endogenous peptides on the nascent HLA class I molecules, additionally expressing costimulatory molecules (CD80 and CD54); unfortunately these insect cells are rather rapidly eliminated at 37°C, resulting in massive release of antigens and need additional feeder cells in order to act as effective APCs [64];

(b) murine 3T3 fibroblast cell line, transduced with CD80 (B7.1), CD54 (ICAM-1) and CD58 (LFA-3) and a selected HLA class I allele [64, 65];

(c) K32, a CD32 (human low affinity Fcγ receptor)-transfected precursor K562 chronic erythroleukemic cell line, constitutively expressing exclusively HLA-C molecules, but also B7-H3, ICAM-1, LFA-3 and showing T cell-dependent upregulation of PD-1L and PD-2L; only after transfection with 4-1BBL and after being coated by HLA class I tetramers or Ig-HLA class I fusion molecules or transfected with HLA class I, CD54, CD58, CD80 and CD83, these cells could then be used as antigen-specific aAPCs [64, 85];

(d) exosomes, which are cell-derived vesicles, about 60–90 nm in size, contained within larger endosomes and secreted into the extracellular space after endosomes fuse with a plasma membrane; they can stimulate immune responses in vivo and antigenic peptides can be directly loaded onto purified exosomes [64];

(e) liposomes, for example composed of cholesterol and a neutrally charged phospholipid phosphatidylcholine as scaffolds accommodating HLA molecules [64];

(f) magnetic beads, coated with Ig-HLA class I fusion molecules and anti-CD28 antibodies [64, 65, 86];

(g) latex microspheres coated with HLA-class I-antigenic peptide complexes or HLA class I-antigenic peptide tetramers, costimulatory molecules CD80, CD83, CD54 and/or anti-CD28 antibody, in the presence or absence of autologous PBMCs as feeder cells [65, 87, 88];

(h) soluble HLA class I-antigenic peptide monomers, cross-linked with anti-CD27 or anti-CD-28 costimulatory antibodies onto a streptavidine core molecule [89].

6.3. Genetically Engineered Antigen-Specific CTLs. An alternative to selecting specific antitumor or anti-pathogen T cell clones from patients, is to engineer such immune reactivity by transferring genes coding for clonally specific $\alpha\beta$TCRs or single-chain chimeric antigen receptors (CARs), therefore obviating the prolonged antigen-driven T cell selection [90]. Genes encoding TCRs can be isolated from pre-selected (capture of IFN-γ secreting $CD8^+$ cells following specific antigenic stimulation or the use of HLA allele-antigenic peptide multimers) high avidity human T cells or T cells from transgenic mice immunised with human cancer antigens, subsequently cloned and inserted in retroviruses [62]. The Vβ usage in selected high affinity clonal TCRs can be defined by TCR Vβ-repertoire analysis, based on a panel of monoclonal antibodies, recognizing approximately 70% of TCR Vβ families, and flow cytometry [91]. Additionally, by performing TCR Vβ spectratyping, that is, multiplex real time PCR (RT-PCR) reactions, T-cell repertoire diversity can be analyzed by comparing the relative frequencies of different clonal length products within the CDR3 region of a particular TCR Vβ family [92]. The genes encoding for high affinity tumour antigen-specific $\alpha\beta$TCRs have been successfully transduced into patients' autologous T cells by using retroviral vectors and were clinically tested [93]. However the production and testing of retroviral producer cell lines and clinically applicable GMP batches of virus remain expensive and time consuming. Additionally, the discovery of transgene-containing lymphoid malignancies in children treated with retrovirally transduced HSC, has discouraged the use of this vector system for genetically manipulated T cell adoptive transfer trials [42]. Therefore the use of naked DNA and electroporation is being studied as an alternative approach, as it has been proven to be safe, reproducible and economically feasible, but may be limited by the time needed for selection of T cells containing stable genomic transgene integrants. It seems that a proper compromise would be to use DNA transposon/transposase elements which can efficiently and safely transfect T cells and can be manufactured under clinically grade GMP conditions [42]. But there are also other pitfalls of $\alpha\beta$TCR gene transfer, for example:

(i) many tumors can evade immune surveillance by down-regulating or completely abrogating MHC expression;

(ii) a detailed knowledge regarding patient's HLA allele specificities is required in order to maintain specific antigen recognition by cloned TCRs;

(iii) the specificity of transferred α and β chain pairs can be altered due to their unwanted pairing with endogenous $\alpha\beta$ TCR chains in the recipient cells, potentially resulting in autoreactive receptors.

To limit the latter mispairing problem, the newly introduced $\alpha\beta$ TCR chains can be fused to CD3-ξ or their constant domains equipped with cysteine residues in order to facilitate additional inter-chain disulfide bond formation. Alternatively their TCR constant domains can be replaced with those of murine origin [42].

By using CAR technology, many pitfalls of $\alpha\beta$TCR transfer, especially those, related to HLA antigen restriction, can be avoided. CARs are constructed out of an antigen-recognizing extracellular domain, in most cases a single-chain antibody fragment (scFv) containing the heavy (V_H) and light (V_L) variable chains, fused to an intracellular T-cell signalling domain, usually the CD3 ξ-chain providing the cell activation signal. Costimulatory molecules such as 4-1BB, OX40 and ICOS can also be added to promote full activation of CAR^+ $CD8^+$ T cells [94]. The use of CAR-transduced $CD8^+$ T lymphocytes seems to be ideal for targeting B-cell malignancies, but clinical studies involving other types of cancer are also being performed [95, 96].

Gene modification can also be used to enhance T-cell function, for example by conferring a helper T-cell independent phenotype to $CD8^+$ T lymphocytes, either with the transfer of chimeric GM-CSF-IL-2 receptor, restoration of CD28 expression or the expression of the catalytic subunit of telomerase, thereby enabling specific antigen-driven autocrine proliferation as well as rejuvenation of the CTL effector pool [46].

7. Optimal Cell Dose for Adoptively Transferred $CD8^+$ T Cell-Based Cellular Products

The optimal or at least the minimal T cell dose to be adoptively transferred into recipient in order to obtain desirable therapeutic effect, are still not known. However, we can speculate that they are dependent on many important factors, such as

(i) manufacturing conditions enabling the production of highly efficient antigen-specific, long-term in vivo persisting effector cells;

(ii) recipient's condition at the time of the adoptive cell transfer (nonmyeloablative lymphodepleting preconditioning is crucial);

(iii) tumor type, tumor size or degree of viremia;

(iv) relative densities and affinities of counterreacting effector cell antigen-specific immunoreceptors (TCRs) and TAA or viral antigens presented as antigenic peptides within the context of recipient's MHC molecules, expressed on target cells [42].

Anyhow, therapeutic effects have been reported for a substantial variety of cell concentrations that were used to adoptively treat different diseases, for example

(i) $0,2 \times 10^5$–1×10^5 CMV-specific T-cell lines/kg, administered in a single infusion [97];

(ii) 5×10^6–1×10^8 multi-virus–reactive cell line/m^2, administered in a single infusion [98];

(iii) 4×10^7–3×10^8 EBV-specific T-cells/m^2, administered in 1 or 2 infusions [99];

(iv) $0,11$–$13,1 \times 10^8$ melanoma-specific T cells/infusion, administered in at least 3 infusions [100];

(v) $2,3 \times 10^{10}$–$13,7 \times 10^{10}$ melanoma-specific T cells/infusion [101];

(vi) $\sim 5 \times 10^{10}$ ex vivo selected and expanded autologous TILs isolated from metastatic melanoma patients/infusion [102].

8. Preconditioning of Patients Receiving Adoptive Transfer of Antigen-Specific T Cells

Numerous animal experiments as well as clinical studies in humans have clearly shown that the effectiveness of adoptively transferred T cells can be augmented when combined with conventional cytotoxic agents such as cyclophosphamide, doxorubicin, paclitaxel, fludarabine and sometimes also with a low-dose irradiation [103]. Therefore the nonmyeloablative lymphodepletion of the host, preceding the adoptive immunotherapy became one of the crucial components of the treatment as it has been proven that it eliminates recipient's tumor-protecting regulatory/suppressor T cells as well as cytokine sinks, that is, lymphocytes that compete with the transferred immune cells for homeostatic γ_c cytokines, such as IL-7 and IL-15 which enhance $CD8^+$ T cell activity [103]. For example, in clinical trials, where the effects of adoptively transferred autologous in vitro expanded TILs, obtained from metastatic melanoma patients, combined with a high-dose of recombinant human IL-2 were studied, the lymphodepletion prior to cell and cytokine infusion was achieved by cyclophosphamide (60 mg/kg during the first 2 days) and fludarabine (25 mg/m^2 during the next 5 days). More than 50% of treated patients experienced an objective clinical response and few of them were considered complete responders [104, 105]. In some patients with a noticeable tumor regression, a large in vivo expansion of transferred antitumor-specific lymphocytes was observed. These $CD8^+$ T cells with a single Vβ7 TCR clonotype persisted in high percentages (>70%), relative to total numbers of lymphocytes, for several months [104]. But regardless of the promising results obtained so far, the optimal lymphodepletion conditioning regimen obviously still has to be defined.

9. Conclusions

Enormous progress in understanding T cell biology and physiology, combined with the evolution of sophisticated technologies for generation, detection, selection, and clonal expansion of MHC class I-restricted antigen-specific CTLs have made the adoptive transfer of these and other immune cells into previously non-myeloablatively pre-conditioned patients, a rather effective clinical procedure with a highly promising perspective. Increasing numbers of clinical studies with encouraging preliminary results are a vivid driving force for the ever-competing research and innovation within this field. Through all this progress it is becoming more and more obvious that in order to be successful, various combined approaches should be used to efficiently modulate immune responses, which is undoubtedly most obvious in antitumor immunotherapy. The fact that only lymphodepleted hosts can benefit from adoptively transferred anti-TAA-specific T cells has clearly shown that besides providing highly specific antitumor responses, deblocking of numerous tumor-protective mechanisms among which some of the most pronounced are sustained by tolerogenic and anergic immune cells, is vital for this kind of therapy to be successful. Another critical issue, crucially related to development of immunotherapy is the obligatory concordance of all procedures involved in the preparation of cellular products, with more and more strict regulations demanding the fulfillment of GMP standards. It seems that the regulative has somehow overtaken the providers of reagents and equipment, therefore creating certain problems resulting in late translation of experimental achievements into the clinical practice. This of course is also a consequence of lacking funds for very costly phase III clinical studies and registration of such cellular drugs.

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