Quality and quantity: new strategies to improve immunotherapy of cancer

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Adoptive immunotherapy is a promising approach for the treatment of infectious diseases and cancer. Several lines of research are currently focusing on the development of different technologies to facilitate the induction and expansion of antigen-specific T cells. Here, we discuss two current articles that affect the field of adoptive immunotherapy. One article describes the engineering of artificial antigen-presenting cells, which promise to replace the cumbersome dendritic-cell approach for the *in vitro* generation of large numbers of antigen-specific T cells. The second development is a description of a new technique for the detection of functionally active antigen-specific T cells, which will enhance the ability to control the quality of the T cells to be used in adoptive immunotherapy. Together, these exciting findings will advance the field of immunotherapy.

Adoptive immunotherapy is an exciting therapeutic technique for the treatment of cancer and infectious diseases that has developed during the past decade. It has been evaluated for the treatment of viral infections, such as cytomegalovirus (CMV) [1,2], Epstein–Barr virus (EBV) [3] and HIV [4], as well as a variety of malignancies, including malignant melanoma [5,6], multiple myeloma [7] and EBV-associated lymphoproliferative disorders and tumors [8–11].

Adoptive T-cell transfer involves the *ex vivo* induction and expansion of antigen-specific autologous T cells over a short period, from low precursor frequencies to clinically relevant cell numbers, followed by re-infusion into patients. Although a variety of different approaches have been used to generate antigen-specific T cells for adoptive immunotherapy, improvements continue to be made on the ability to generate antigen-specific cytotoxic T lymphocytes (CTLs) that efficiently recognize target cells. These approaches increase both the yield and specificity of the T cells, in addition to minimizing the *in vitro* culture time and expense necessary to obtain functional T cells, which can be further expanded *in vivo*. Previous pivotal improvements include optimizing the isolation of lymphocytes from patients, the culture conditions, the methods used for the induction and expansion of T cells and the quality of the resulting CTLs [12–15]. In addition, new approaches have augmented the ability to accurately assess the T cells at every stage. Together, these crucial advances have helped researchers explore the use of *in vitro*-generated CTLs for adoptive immunotherapy.

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Obstacles in the field

One of the most widely used approaches for the induction and expansion of antigen-specific CTLs has been based on the use of autologous antigen-loaded dendritic cells (DCs) as antigen presenting cells (APCs). However, the generation and maintenance of DCs is expensive and cumbersome. The labor involved, together with the cost of the cytokines needed to differentiate and mature the DCs, leads to total expenditures of $30 000 or more per patient. Furthermore, variability in DC number and the quality of the DCs are affected by both pretreatment of the patient and the patient's disease, such as multiple myeloma, breast cancer and prostate cancer [16–19]. Therefore, although multiple leukaphareses (preparations of leukocyte concentrates, from which the red cells and leukocyte-poor plasma are returned to the donor) are necessary to generate sufficient numbers of DCs, the quality of the DCs that are obtained is still highly variable, reducing the ability to reliably deliver a therapeutic dose of T cells to the patient. Another problem is that the tumor-specific T cells that are found in patients with cancer are often dysfunctional [20]. Although these patients have relatively high numbers of antigen-specific T cells, as determined by HLA-tetramer or -dimer analysis (Box 1), which indicates that they have undergone previous clonal expansion, these

Box 1. Multimeric peptide–MHC complexes can be used to visualize antigen-specific T cells

Soluble, multimeric peptide–MHC molecules have facilitated the ability to detect and characterize antigen-specific T cells by flow cytometry [20,29,30]. These multimerized MHC molecules display significantly increased avidity for their specific TCR, because they bind with multiple arms independently to the same cell.

Most commercially available and widely used multimeric peptide–MHC molecules are dimeric and tetrameric MHC molecules. MHC-dimer molecules have been developed in which the extracellular portion of the MHC molecule (human HLA-A2) is fused to the constant region of an immunoglobulin heavy chain protein (mouse IgG_1). In contrast to the tetrameric MHC molecules, which are produced biochemically [29], dimeric MHC–IgG fusion proteins are expressed in eukaryotic cells, so that subsequent denaturing and refolding of the MHC molecules is not necessary. Peptide–MHC–Ig complexes can be used to identify antigen-specific T cells as originally described by Greten *et al.* [30].

Altman *et al.* first described tetrameric peptide–MHC complexes, which are produced by combining soluble peptide–MHCs tagged with biotin and bound by fluorescently labeled streptavidin. These molecules have been successfully used to directly visualize antigen-specific T cells. The use of tetramer technology has recently been reviewed [31,32].

T cells are defective in their ability to produce interferon-γ (IFN-γ) and cannot lyse target cells upon stimulation. Thus, they cannot target and destroy tumor cells, as would be expected if the T cells were functional. Because other antigen-specific CTL populations in the same patient function normally, this is strong evidence for anergy of the tumor-specific T cell population.

Taken together, these issues underscore the importance of the development of technologies to generate clinically relevant numbers of highly specific and reactive cytotoxic T cells without depending on the use of autologous DCs. Furthermore, it is particularly important to overcome the tolerance and/or anergy of naturally occurring tumor-specific, vaccination-induced T cells or the adoptively transferred cytotoxic T cells.

In this article we describe two recent developments that advance the field of adoptive immunotherapy: (i) the use of artificial antigen-presenting cells (aAPCs) for the *in vitro* generation of antigen-specific T cells and (ii) the use of CD107a as marker for the detection of functionally active antigen-specific T cells.

HLA–Ig-based aAPCs: a new player in the field

Efficient T-cell activation requires the delivery of a combination of signals, through the engagement of the T-cell receptor with antigen–MHC (major histocompatibility complex) complexes (signal 1) and co-stimulatory molecules (signal 2), such as the interaction between CD28 (which is expressed on T cells) and B7 (which is expressed on APCs). June *et al.* were the first to develop a non-specific aAPC for the expansion of T cells, which was based on magnetic beads coated with antibodies against CD3 and CD28 [21]. Although the use of non-specific aAPCs was useful for the expansion of $CD4^+$ T cells, it has not been efficient for long-term expansion of $CD8^+$ T cells [22] or for the maintenance of their specificity. Consequently, an aAPC was developed in which HLA-A2–immunoglobulin (Ig; signal 1) and an anti-CD28 antibody (signal 2) were coupled to the surface of a cell-sized magnetic bead [23,24]. This system aims to control and modulate the nature and ratios of signals 1 and 2 for the optimal induction and expansion of peptide-specific CTLs. Preliminary studies have investigated this by analyzing the ability to generate CTLs directed against the CMV virus-antigen CMV_{pp65}, as well as the tumor antigens Mart-1 (a self antigen) and NY-ESO (a cancer– testis antigen). Comparisons of aAPCs with DCs showed that the aAPCs were better than DCs at inducing and expanding antigen-specific CTLs, which were directed against various antigenic peptides, from multiple donors. Using this approach, starting with only 10^6 $CD8^+$ T cells isolated from fresh blood, up to 10^9 T cells with a specificity of $>85\%$ were obtained in less than two months. The CTLs that were obtained recognized and lysed human cells that expressed the endogenous antigen targets at low densities on their surface [23]. These data indicate that aAPCs can stimulate the production of large numbers of highly efficient antigen-specific CTLs.

Although only one prototype aAPC has been studied, this approach can be used to vary the concentration of costimulatory molecules and/or alter the ratio of MHC–peptide:costimulatory complexes in the preparation. Thus, T cells with different biological functions could be developed, which would enable adoptive immunotherapy approaches to treat a variety of different diseases. Furthermore, although the HLA-A2 haplotype was chosen because of its high incidence in the general population (therefore enabling the development of therapeutic options for nearly half of the patient pool), the simple addition of a few more haplotypes could provide therapeutic options for over 90% of the population. In addition, the generation of HLA class II-based aAPCs, which are similar to those described by Maus *et al.*, or an HLA class II–Ig-based aAPC that is analogous to the aAPC described here, could be useful in generating antigen-specific $CD4^+$ T-helper cells. This would enable the adoptive T-cell transfer of antigen-specific $CD4^+$ and $CD8^+$ T cells for a more complete immune reconstitution.

There are several advantages of the aAPC approach: (i) patients do not have to undergo multiple leukaphereses; one simple blood draw will be enough to grow sufficient numbers of antigen-specific T cells; (ii) the stimulation of T cells with aAPCs is not dependent on the state and availability of the patient's DCs, a crucial parameter in immunosuppressed cancer patients; (iii) the peptide loading of aAPCs can be controlled to ensure that only the peptide of interest is presented and not a random assortment of peptides in addition to the desired one, as is the case for DCs; and (iv) this 'off-the-shelf' aAPC, which can be produced using good manufacturing practice (GMP) standards, can be readily developed for the translation of adoptive immunotherapy into clinical trials.

CD107a: A new referee on the field

In addition to developing technologies for expanding cells, it is crucial to explore the functionality of the expanded T cells. Researchers need to focus on the antigenic specificity, function, homing and proliferation potential of the T cells (i) before the start of the culture, (ii) for quality control during the maintenance period, (iii) shortly before adoptive transfer into the patient and (iv) after transfer, for long-term monitoring.

A variety of direct and indirect methods exist to determine the antigenic specificity of CTL populations. Although T cells can be assayed for their specificity using direct dimer or tetramer staining (Box 1), conclusions cannot be made about the functionality of the T cells. Alternatively, several indirect assays exist to determine the frequency of antigen-specific CTLs, based on their effector function, such as the IFN-γ assay (capture assay), the ELISpot assay and the intracellular cytokine-secretion (ICS) assay. However, there are several disadvantages associated with these assays. The IFN-γ-capture assay requires large numbers of cells, and the ELISpot assay takes several days to complete and enables only semi-quantitative analysis. Furthermore, T cells are dead after performing either an ICS or ELIspot assay and, therefore, cannot be used for additional experiments. These assays are also difficult to perform, time and cell consuming, and the secretion of cytokines does not reveal anything about the ability of the T cells to lyse and efficiently kill targeted cells. As a result, the ^{51}Cr-release assay remains the standard method for determining cytotoxicity; however,

the lysis does not always correlate with other effector functions [25–27]. Therefore, although a variety of methods exist to analyze T cells in terms of peptide specificity and functionality, there is still the requirement for a link between the direct methods, such as the ICS and capture assays, and the indirect methods, including ^{51}Cr release.

A recent paper by Rubio *et al.* described the use of a flow-cytometry-based assay to simultaneously analyze antigen-specific CTL frequency and the ability of these cells to kill a tumor target [28]. They reported that the expression and surface mobilization of CD107a correlates with the ability of the CTLs to kill their target. CD107a (LAMP-1) is a glycoprotein that is associated with the cytolytic-granule membrane. Upon activation, cytolytic granules fuse with the cell membrane, releasing the toxic substances that are stored within the granules, such as perforin and granzymes, and kill target cells. The new procedures directly measure degranulation by determining the cumulative amount of granular membrane proteins, such as CD107a, on the cell surface of responding antigen-specific T cells. Significant expression of cell-surface CD107a can be detected within 30 min following the stimulation of primary $CD8^+$ T cells, and reaches its maximum by 4 h. This appears to be the only assay that delivers the combined information of a direct (tetramer analysis) and indirect (lysis assay) method. If these were coupled with a cytokine-production assay, it would enable the simultaneous assessment of both CTL-effector functions and TCR specificity in one assay.

This technique enables a fast, functional analysis of living cells. Furthermore, antigen-specific CTLs could be sorted specifically and expanded using peptide-loaded HLA–Ig-based aAPCs. This approach might increase the efficiency of culture, because the T cells are already selected for their lytic capability. The additional information provided by this assay can be used to further improve therapeutic regimens for vaccination and adoptive transfer.

The future: how to beat the opponent

Although methods have been developed to overcome many barriers, some parts of the ideal adoptive immunotherapy for cancer and infectious diseases remain incomplete. Comparison with DC-induced CTLs has indicated that *in vitro* aAPC-induced CTLs are functionally similar to their DC-induced counterparts, but this still needs to be confirmed *in vivo*. The ability to control the quality and sequence of stimulation by designing aAPCs with variations to signal 1 and different types of signal 2 also offers the potential of converting tolerant T cells from patients into functional effector T cells with the ability to kill tumor cells or virally infected cells. This could be monitored using the new flow-cytometry-based technique established by Rubio *et al.*

The distinct advantage of HLA–Ig based aAPCs, and the reason why it holds promise for the future, is because the technique is simple, reproducible and versatile. Furthermore, the ability to modify aAPCs, by changing the ratio of HLA–Ig:antibodies against CD28, or by introducing other co-stimulatory molecules, such as 41-BBL, enables the control of the effector function of the resulting T-cell population. Together with the use of CD107a for T-cell analysis, this technique offers significant potential for the treatment of cancer, where the issue of T-cell recognition and tolerance and/or anergy is crucial, and has been a major impediment to tumor immunotherapy. This technique offers the possibility of identifying tolerogenic T cells by using CD107a in combination with HLA-tetramer or -dimer technology, as well as the removal of T-cell tolerance through the use of custom-made aAPCs for T-cell stimulation and expansion.

Concluding remarks

Two new technologies promise to revolutionize approaches to immunotherapy. The HLA–Ig based aAPC is a general platform technology that could enable investigators to introduce antigenic specificity and co-stimulatory complexes for the production of peptide-specific CTLs. The use of CD107a to analyze the resulting T cells combines several standard assays for probing CTLs in one simple method. This facilitates and improves the monitoring and outcome prediction for adoptive immunotherapy. Together, these recent developments increase the likelihood that adoptive immunotherapy will soon emerge as a significant part of our armamentarium for the treatment of infectious diseases and cancer.

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Letter

Darwinism and pharmacogenomics: from 'one treatment fits all' to 'selection of the richest'?

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Pharmacogenomics and pharmacogenetics are relatively new fields, and have arisen from recent advances in genetic research. They offer new perspectives on the development of pharmaceuticals, allowing drug design to be targeted specifically to the genotype of selected populations. The discussion of who will be considered for the development of these tailored drugs and who will be excluded, in a situation in which both research resources and public expenditure are limited, is provoking and has led to several, still unanswered ethical questions and concerns about fairness and the potential discrimination of fringe groups.

Based on the statistical analyses of population averages, patient groups are generally assumed to be homogenous. Treatment is generally given according to empirical, if not arbitrary, guidelines, often ignoring potential discrepancies in drug response. Drug therapy is targeted, therefore, to the broadest possible patient population. In view of the increasingly aging population in the western world, socioeconomic expenses for both treatment and adverse drug reactions are booming, whereas public spending is progressively under pressure. Disparities in responses to drugs are common among patients and represent a significant clinical problem. These disparities might, at least in part, result from genetic variations among individuals or populations. A drug that is well tolerated and elicits a good response in some patients might be ineffective, toxic or cause severe adverse events in others. Drug response might, of course, also depend significantly on the underlying condition being treated and could be influenced by concomitant medications and drug interactions, by patient age, sex, organ function, lifestyle, education, socioeconomic status, environmental factors and accompanying illnesses. However, many of these factors are difficult to control for and are likely to be affected by a person's ethnic background [1,2].

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