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Chapter 6

Immunotherapy through TCR gene transfer

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Immunotherapy through TCR gene transfer

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The antigen specificity of T lymphocytes is dictated solely by the T cell receptor (TCR) \( \alpha \) and \( \beta \) chains. Consequently, genetic transfer of TCR chains may be an appealing strategy with which to impose a desirable virus- or tumor-antigen specificity onto cytotoxic or helper T cell populations. We describe here the genetic introduction of a virus-specific TCR into peripheral T cells in a mouse model system. These experiments showed that T cells redirected by TCR gene transfer expanded upon viral infection of mice and efficiently homed to effector sites. In this setting, TCR gene transfer was not associated with any significant autoimmune pathology. In addition, small numbers of TCR-transduced T cells promoted the rejection of antigen-expressing tumors in vivo. These data suggest that the redirection of T cells by TCR gene transfer is a viable strategy for the rapid induction of virus- or tumor-specific immunity.

The role of adaptive immunity in controlling tumor growth has become increasingly well defined. Among patients with acquired or congenital immune deficiency the incidence of tumors with a viral etiology, such as Epstein-Barr virus (EBV) lymphoma, is markedly increased. Likewise, in mouse models for immunodeficient states, tumor incidence is greatly increased not only for carcinogen-induced tumors but also for tumors that spontaneously arise. These latter studies showed that tumors that arise in immunocompetent hosts are selected for a low immunogenicity towards the host’s immune system. This selection of tumor cells whose antigens are not appropriately recognized by the host’s immune system suggests that curative vaccination approaches to improve tumor-specific T cell immunity may have inherent limitations. In line with this, the most successful form of tumor-specific immunotherapy to date has been the infusion of allogeneic donor lymphocytes for tumors such as chronic myelogenous leukemia, or the use of virus-specific T cell lines in the case of EBV lymphoma.

Despite their success, these treatment options have mostly remained experimental. This is because of the sometimes severe graft-versus-host disease (GVHD) upon allogeneic lymphocyte infusion and the difficulties in finding suitable human leukocyte antigen (HLA)-matched donors. An alternative strategy to harness the T cell system for immunotherapeutic purposes would be the alteration of T cell recognition by genetic means. Transfer of the genes encoding the T cell receptor (TCR) \( \alpha \) and \( \beta \) chains (TCRA and TCRB, respectively) into peripheral blood T lymphocytes could enhance antigen-specific immunity by increasing the frequency of pathogen- or tumor-specific T lymphocytes. In the first TCR gene-transfer experiments, the transfer of TCRA and TCRB genes sufficed to impose the major histocompatibility (MHC)-restricted antigen specificity of a T cell onto the recipient T cell. In subsequent in vivo studies, virus and tumor-specific TCRs were functional upon genetic transfer of TCR chains into both human and murine T cells.

We analyzed the in vivo feasibility of TCR gene transfer with respect to antigen-driven expansion and homing of TCR-transduced T cells. We also examined the autoimmune pathology that may possibly occur as a side effect of the gene transfer as well as the in vivo eradication of established tumors by redirected T cell populations. Our results establish TCR gene transfer as a means to rapidly induce profound T cell immunity in vivo.

Figure 1. Genetic transfer of TCR chains resulted in expression of functional TCRs. (a) Flow cytometric analysis of total splenocytes 3 days after retroviral transduction with FS TCRA and TCRB genes or mock-transduction. Cells were stained with PE-anti-CD8 and allophycocyanin-NP-tetramer. Upon TCR-transduction, 6.7% of CD8+ T cells were NP-tetramer+. Tetramer staining was done at room temperature. (b) Intracellular IFN-γ staining of FS TCR-transduced and mock-transduced splenocytes. Cells were incubated in the presence of NP366-374 (closed circles) or control A/HKx31 NP peptide (open circles) stained with PE-anti-CD8, permeabilized and stained with FITC-anti-IFNγ. Data are means±s.d. of triplicate experiments.

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Results
Transfer of T cell specificity by TCR transfer

To study the feasibility of TCR gene transfer in a mouse model we used the F5 TCR. The F5 TCR recognizes the immunodominant H-2D^D-restricted cytotoxic T lymphocyte (CTL) epitope that consists of amino acids 366-374 of the influenza strain A/NT/60/68 nucleoprotein, referred to hereafter as NP(366-374)\(^1\). Both TCR\(^A\) and TCR\(^B\) were inserted into a Moloney-based retroviral vector\(^2\); the resulting retrovirus (pMX-F5) was used for TCR gene transfer by means of retroviral infection in short-term ex vivo cultures.

After the introduction of F5 TCR chains into mouse splenocytes by retroviral infection, 5–15% of the total CD8\(^+\) T cells expressed the introduced F5 TCR, as determined by MHC tetramer flow cytometry. In contrast, no CD8\(^+\) T cells that were positive for the A/NT/60/68 NP(366-374) H-2D^D-tetramer (referred to as NP-tetramer hereafter) were observed in mock-infected cultures (Fig. 1a) or cultures infected with a control virus (data not shown). F5 TCR-transduced T cell cultures showed a pronounced NP(366-374)-specific effector function without any requirement for previous antigen encounter, as measured by intracellular interferon-\(\gamma\) (IFN-\(\gamma\)) staining (Fig. 1b). This indicated that retroviral introduction of TCRs could lead to the rapid generation of antigen-specific T cell immunity.

TCR-transduced T cells expand in vivo

Subsequently, the feasibility of TCR gene transfer in an in vivo model system was addressed (Fig. 2). Mouse splenocytes that were either mock- or F5 TCR-transduced were reinjected into mice before influenza A infection. To distinguish between introduced and endogenous cell populations, donor and recipient mice were mismatched for the allele type marker Ly5.5 (CD45). CD8\(^+\) cells that lacked this allele type marker were not detected in naïve or influenza A-infected Ly5.1 mice that had not received lymphocyte infusions (data not shown). Mock-transduced (1.8x10\(^7\)) or F5 TCR-transduced (1.8x10\(^7\) Ly5.2\(^+\) splenocytes were injected intravenously into Ly5.1 hosts; of the CD8\(^+\) T cells within this population, 9.2\% (2.7x10\(^6\) cells) were NP-tetramer\(^+\).

Two days after cell transfer, mice were challenged with either influenza A/NT/60/68 virus or a control influenza A virus (A/HKx31) by intranasal infection. Two days after viral infection, a time-point at which virus-specific T cell immunity is still below detection levels during influenza A-induced pneumonia\(^3^4\), low to undetectable numbers of introduced CD8\(^+\) T cells were present in all mice; this occurred independently of the type of cells administered (mock-transduced or F5 TCR-transduced) or the influenza A strain used for infection (Fig. 2a).

In contrast, during the subsequent course of viral infection, a prominent expansion of F5 TCR-transduced CD8\(^+\) T cells was observed in all A/NT/60/68 influenza A-infected mice that received F5 TCR-transduced cells (Fig. 2a,b). This T cell expansion was a direct consequence of signaling through the introduced F5 TCR, as no expansion was observed in A/NT/60/68 influenza A-infected mice that had received mock-transduced cells. Likewise, no proliferation of TCR-transduced cells was observed in mice that received TCR-transduced splenocytes but were infected with an irrelevant influenza strain, which indicated that the expansion of TCR-transduced cells was fully antigen specific.

Quantification of the expansion of TCR-transduced T cells over the course of influenza A/NT/60/68 infection showed a >1000-fold increase in the number of F5 TCR-transduced cells. Because a substantial fraction of effector cells undergoes apoptotic cell death in the course of a cytotoxic T cell response\(^5\), this number may have been underestimated. After viral clearance, both the TCR-transduced and endogenous antigen-specific T cell populations decreased to low numbers in peripheral blood, which was consistent with previous analyses of T cell memory formation\(^6\).

To determine the homing pattern of TCR-transduced cells, the distribution of redirected T cells was compared to that described for regular
T cell responses upon influenza A infection\textsuperscript{13,14}. Consistent with the known distribution of influenza A-specific T cells, large numbers of TCR-transduced T cells were observed in the spleen, inflamed lung tissue and the draining (mediastinal) lymph node during acute influenza A pneumonia (Fig. 3). Collectively, these data showed that genetic transfer of TCRs into peripheral cells resulted in the redirection of the antigen specificity of CD8\textsuperscript{+} T cells, as documented by their response to viral infection \textit{in vivo}.

Retroviral transductions were done on total spleen cells, including CD4\textsuperscript{+} T cells. Upon transduction with the pMX-F5 virus, 5–10\% of the CD4\textsuperscript{+} T cells expressed the F5 receptor, as shown by MHC-tetramer staining (data not shown). However, in contrast to the marked expansion of F5 TCR CD8\textsuperscript{+} T cells upon influenza A/NT/60/68 infection, no expansion of F5 TCR CD4\textsuperscript{+} T cells was detected (data not shown). This lack of expansion of TCR-transduced CD4\textsuperscript{+} T cells was consistent with the idea that CD4 coreceptor binding and signaling is required for proper T cell activation; it suggests that, for the simultaneous induction of CD4\textsuperscript{+} T cell immunity, coapplication of retroviruses encoding MHC class II-restricted TCRs may be considered.

Although no proliferation of TCR-transduced CD4\textsuperscript{+} T cells was observed, it was remained possible that these or other retrovirally transduced non-CD8\textsuperscript{+} spleen cells contributed to the development of immunity upon TCR gene transfer. To address this issue, CD8\textsuperscript{+} cells were isolated after transduction with pMX-F5 retrovirus or a control virus (pMX). All mice that received F5 TCR-transduced CD8\textsuperscript{+} T cells showed large expansions of infused CD8\textsuperscript{+} T cells, which occurred to a similar extent (Fig. 2c) and with the same kinetics that were observed upon infusion of total TCR-transduced splenocytes. In mice that received CD8\textsuperscript{+} T cells with the control retrovirus, no expansion of infused CD8\textsuperscript{+} cells was observed. In addition, in mice that had received TCR-redirected T cells, the endogenous influenza A/NT/60/68-specific T cell response was suppressed (Fig. 2c), and the extent of this suppression was inversely correlated with the magnitude of the exogenous T cell response (data not shown). This suppression likely resulted from competition for NP(366-374)-expressing antigen-presenting cells (APCs) between the TCR-transduced and endogenous T cells\textsuperscript{15}. This provided further evidence for the proper \textit{in vivo} function of redirected T cell populations.

**Side effects of TCR gene transfer**

Infusion of allogeneic lymphocytes in patients and in mouse model systems frequently results in severe acute GVHD\textsuperscript{4}. Likewise, the transfer of TCRs into (autologous or allogeneic) lymphocytes could conceivably be complicated by autoimmune pathology, for example through the action of mixed TCR dimers with unpredictable specificity that are formed by assembly of endogenous and exogenous TCR chains. To assess the possible induction of autoimmunity as a result of TCR gene transfer, a full pathology survey of four mice was done 56 days after TCR transfer.

Evidence of any significant autoimmune pathology in any organ was limited or absent in all mice examined. Like many untreated mice, two of four mice showed signs of mild gastritis that consisted of local leukocyte infiltrations without any signs of supplicative inflammatory processes or tissue degeneration. Three mice showed a subacute middle ear infection (otitis media), which was also observed in 50% of influenza A–infected control mice, which was consistent with the role of influenza infection in the development of middle ear infection in humans and in animal models\textsuperscript{15,16}. Classical target organs of GVHD, such as skin and liver, were not affected in any of the animals. Thus, even though these mice had experienced large expansions of the TCR-redirected T cells, autoimmune pathology induced by these cells was limited and compared favorably to the autoimmune pathology induced by immune interventions such as allogeneic lymphocyte infusion.

**TCR-transduced cells promote tumor eradication**

The redirection of the specificity of T cell populations by the introduction of TCR chains may be particularly useful for inducing tumor-specific T cell immunity in immunodeficient states in which allogeneic T cell infusions have been successful. To determine the value of TCR-transduced lymphocytes in tumor eradication in an immunodeficient context, we performed a lympholytic assay using tumor-bearing mice, which were injected with tumor cells in vivo and then treated with TCR-transduced and endogenous T cells. The results showed that TCR-transduced T cells were able to eradicate tumor cells efficiently, highlighting the potential of this approach for cancer immunotherapy.

**Immunotherapy through TCR gene transfer**

\textit{Immunotherapy through TCR gene transfer}\n
**Figure 3. Distribution of expanded TCR-transduced cells.** Flow cytometric analysis of spleen, lung and draining (mediastinal) lymph node cells of Ly5.1 mice, which received 3x10\textsuperscript{5} F5 TCR-transduced Ly5.2 splenocytes (of which 5x10\textsuperscript{4} were Ly5.2-CD8\textsuperscript{+} T cells), isolated 5 days after infection with influenza viruses A/NT/60/68 or B/Lee/40. Staining of introduced Ly5.1 cells with PE-anti-CD8 and allophycocyanin-NP-tetramers is shown.

**Figure 4. In vivo anti-tumor activity of TCR-transduced cells.** RAG1-129X1Ev mice were injected subcutaneously with 1x10\textsuperscript{4} EL.4 tumor cells. Four days later (indicated by arrows) mice received either F5 TCR-transduced (closed circles) or mock-transduced (open circles) 129X1Ev splenocytes. (a) Tumor sizes and (b) the percentages of NP-tetramer-reactive CD8\textsuperscript{+} cells of total blood cells were measured. Data are means\textpm{}SEM from groups of six mice. (c) Survival of tumor-inoculated mice that had received either TCR-transduced (closed circles) or mock-transduced (open circles) splenocytes.
setting, recombination-activating gene 1-deficient (RAG-1-) mice that did not contain endogenous T and B cell populations were subcutaneously injected with 1×10^6 EL4^+ tumor cells, which express NP366-374 (Fig. 4). In nontreated mice, tumor inoculation led to uncontrollable tumor growth and subsequent death after 3–4 weeks (data not shown). In this model, infiltration of unmodified splenocytes (1×10^6) on day 4 after tumor inoculation resulted in a delay in tumor growth and subsequent long-term survival in 33% of animals (Fig. 4a, c). This partial tumor regression was not accompanied by any visible expansion of A/NP68-specific T cells (Fig. 4b) and likely involved recognition of other tumor antigens or minor histocompatibility antigens. When 1×10^6 TCR-transduced splenocytes were infused—of which only a small fraction (2×10^4) consisted of NP-gated CD8^+ cells—rapid eradication of tumors was observed in all mice (Fig. 4a). In addition, in all cases this tumor clearance was accompanied by a prominent expansion of NP366-374-specific CD8^+ cells (Fig. 4b) and long-term survival of these mice was 100% (Fig. 4c). These data directly showed that small numbers of TCR-transduced T cells could strongly promote tumor eradication in vivo.

Long-term maintenance of TCR-transduced T cells was studied by MHC-tetramer staining and TCR V_p staining 81 days after initial tumor inoculation. In four of four mice, TCR-transduced T cells could still be detected in peripheral blood (1.4±0.3% of CD8^+ T cells). In addition, upon rechallenge of these mice with antigen-expressing tumor cells a rapid increase in the frequency of these cells was observed (9.0±1.8% of CD8^+ T cells on day 6 after tumor challenge) and no expansion of tumor growth was observed. These data showed that the survival and antigen-reactivity of TCR-transduced T cells in immune-deficient mice was long lasting.

**Discussion**

Our data show that T cells in which the specificity has been redirected by TCR gene transfer show all the characteristics of T cell immunity in vivo. The T cell responses of redirected T cells were antigen-specific, and, at least in this model, TCR gene transfer was achieved without the development of any overt signs of autoimmune disease. Although with this retroviral system transduction efficiencies were low (5–15%), the fact that only relatively small numbers of TCR-transduced T cells were infused was more than compensated for by the profound expansion of these genetically modified cells upon in vivo antigen encounter.

Although TCR-transduced T cells survived for extended periods in immunodeficient mice, in wild-type mice T cell memory was dominated by endogenous T cell populations (H. W. H. Kesters et al., unpublished data). Whether this dominance was due to immunogenicity of retrovirally transduced T cells or was a consequence of the degree of exogenous TCR expression remains to be established. The observation of avidity maturation of T cell responses upon secondary antigen encounter^11 coupled to the established sensitivity of Moloney-based vector systems to promote silencing in vivo^10 may argue for the latter possibility. Although for many applications of redirected T cells long-term T cell memory may not be required, it will be useful to establish the effects of TCR transfer with improved viral delivery systems that are less sensitive to transcriptional silencing^12.

Approaches for altering T cell specificity by genetic introduction of CD3_ε fusions with single-chain fragments of TCR or antibody variable regions have been developed^13–15. However, the observation of an optimal dwell time for TCR-MHC interactions during T cell activation^16,17, and the fact that T cell responsiveness is dependent on the interaction of membrane proximal TCR domains with CD3 components^18–20, may argue against the use of such chimeric receptors. In addition, gene transfer of full-length TCR chains should minimize the immunogenicity of introduced receptors. TCR gene transfer should provide an efficient strategy with which to impose a desirable pathogen- or tumor antigen-specificity on peripheral T cell populations via the transfer of naturally occurring tumor antigen- or virus-specific TCRs, as described here. In addition, for tumor lineage antigens for which no high affinity TCRs are present in vivo due to self-tolerance, TCR gene transfer may be used to introduce tumor-specific TCRs that have been optimized in vitro by either yeast or retroviral TCR display^21–23. Alternatively, tumor-specific TCRs may be obtained from HLA-mismatched individuals or from HLA-transgenic mice for which the peptide-MHC complex is nonself.

Several aspects of TCR transfer may be further optimized. For example, in the experiments described here, no attempt was made to limit the formation of TCRoφ heterodimers that consisted of endogenous and exogenous chains. Such newly generated heterodimers may convey new and unpredictable specificities on TCR-transduced T cells that could potentially be autoreactive. Ongoing experiments suggest that the remodelling of the TCRp interface may provide a viable strategy for preventing the formation of such mixed dimers (H. W. H. Kesters and T. N. M. Schumacher, unpublished data), thereby maximizing expression of the introduced TCR and further reducing the risk of autoimmunity. Remodelling of the structurally related immunoglobulin constant domain presents an elegant proof of this principle^22. In addition, coexpression of immunomodulatory genes may be used to genet-
ically tailor properties such as T cell homing behavior or effector function. Analysis of chimeric receptor expression on human tumor cells has suggested a role for these receptors in the metastatic pattern of melanoma and breast cancer cells^23. The introduction of such receptors into T cells could possibly be used to promote T cell migration to the very same metastatic sites.

**Methods**

**Preparation of H-2D^d tetramers and retroviral construct.** Peptides were produced with standard fluoride methods (Chemicon, Temecula, CA). Soluble dihydrocyclohexyl-labeled H-2D^d tetramers were produced as described^24. Bone marrow from an 8- to 10-week-old female, 129/SvJ background, strain 1.5×10^6 cells were incubated for 24 h with NP366-374 peptide (10 μg/ml) and an equal number of unlabeled cells were mock treated. The cell mixture was washed three times in cold RPMI 1640 medium before cell lysis. The supernatant was collected and used to transduce a retroviral vector containing the H-2D^d tetramer-binding region in its envelope and TCRα/p chain expression cassette in order to produce a mature recombinant H-2D^d tetramer. The Tetramer-binding p chain was transduced into EL4^+ tumor cells via retrovirus transduction.

**Preparation of HLA tetramers and retroviral construct.** Peptides were produced with standard TLA methods (Immunotools, Friesoythe, Germany). Soluble dihydrocyclohexyl-labeled H-2Kb tetramers were produced as described^25. Bone marrow from an 8- to 10-week-old female, 129/SvJ background, strain 1.5×10^6 cells were incubated for 24 h with NP366-374 peptide (10 μg/ml) and an equal number of unlabeled cells were mock treated. The cell mixture was washed three times in cold RPMI 1640 medium before cell lysis. The supernatant was collected and used to transduce a retroviral vector containing the H-2Kb tetramer-binding region in its envelope and TCRα/p chain expression cassette in order to produce a mature recombinant H-2Kb tetramer. The Tetramer-binding p chain was transduced into EL4^+ tumor cells via retrovirus transduction.

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Flow cytometry analysis. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-TCR (ab 9099, Becton Dickinson) and anti-mouse CD45.1 (clone Ly5.1, Clone 104, BD Biosciences). Subsequently, cells were cultured for 2 days in Iscove's modified Dulbecco's medium with 10% FCS and recombinant human IL-2 (50 U/ml, PharMingen). For intracellular IFN-γ staining, spleen cells were incubated in the presence of different peptides, Phycoerythrin-conjugated anti-CD8.2 (clone 650E7, BD Biosciences) or MHC tetramers at 37 °C for 15 min. Propidium iodide (1 µg/ml, Sigma) was included before analysis. For flow cytometry analysis, supported by the Dutch Cancer Society (NK.I.97.1442) and the Netherlands Organization for Scientific Research (NWO) (power grant 00-03).

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