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Selective Modification of Antigen-Specific T Cells by RNA Electroporation

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Abstract

It has been observed that the efficient transfection of T cells by RNA electroporation requires prior activation of T cells with mitogens or by anti-CD3 antibody stimulation. We hypothesized that this requirement for T cell activation could be leveraged to express marker genes within activated T cells responding to antigen-pulsed dendritic cells and allow for the selective enrichment and modification of antigen-specific T cells. Using electroporation of mRNA encoding green fluorescent protein as a marker gene, we demonstrate that RNA electroporation can efficiently allow for the separation of cytomegalovirus-specific CD8⁺ and CD4⁺ T cells from bulk culture responding to cytomegalovirus pp65 antigen-pulsed dendritic cells. Furthermore, we demonstrate that cytomegalovirus-specific T cells can be functionally modified by RNA transfection of the C-X-C chemokine receptor, CXCR2, to migrate efficiently toward a variety of CXCR2-specific chemokines *in vitro* and *in vivo*. These studies demonstrate the utility of RNA transfection as a simple method by which to purify and selectively modify the function of antigen-specific T cells for use in adoptive immunotherapy, and importantly provide evidence that transient expression of proteins by RNA transfection is an efficient means of modulating the *in vivo* function of activated T cells.

INTRODUCTION

ADOPTIVE IMMUNOTHERAPY (AIT) with antigen-specific T lymphocytes is a promising treatment for neoplastic and viral diseases (Gattinoni *et al.*, 2006; Lucas and Sun, 2006). The efficacy and clinical applicability of adoptive immunotherapy have been demonstrated in several clinical studies. The transfer of cytomegalovirus (CMV)-specific T cells has been used to safely and successfully protect against CMV reactivation in myelodepleted bone marrow transplant patients, as well as to eliminate active viral disease in immunosuppressed patients (Riddell and Greenberg, 1995; Walter *et al.*, 1995; Numazaki *et al.*, 1997). Moreover, AIT has proven to be highly effective against Epstein–Barr virus-associated lymphoproliferative disease and lymphoma (Rooney *et al.*, 1998; Gottschalk *et al.*, 2005; Cho *et al.*, 2006).

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Dudley and colleagues demonstrated that the adoptive transfer of tumor-specific lymphocytes into lymphodepleted hosts led to the *in vivo* expansion and persistence of these cells and objective clinical regressions of widespread metastatic disease in more than 50% of treated patients (Dudley *et al.*, 2002; Dudley and Rosenberg, 2003; Robbins *et al.*, 2004; Rosenberg and Dudley, 2004; Huang *et al.*, 2005; Gattinoni *et al.*, 2006). These results clearly demonstrated the capacity for adoptive immunotherapy to modulate the progression of advanced solid tumors. The modification of lymphocytes by gene transfer before infusion into patients holds the potential to increase the potency of T cell effector mechanisms and augment therapeutic responses to adoptive immunotherapy (Rischer *et al.*, 2004; Dotti and Heslop, 2005). It has been demonstrated that retrovirus-modified T cells with genetically engineered T cell receptor specificity against a melanoma antigen are able to achieve long-term engraftment within the treated hosts and induce objective clinical regression of widespread metastatic disease in treated patients (Morgan *et al.*, 2006; Zhao *et al.*, 2006a). The modification of T cell functions by gene transfer, therefore, holds the significant potential for improving current adoptive immunotherapy approaches, but the practical application of this process remains significantly challenging. Retroviral vectors, which have been used most frequently for the modification of T cells in adoptive immunotherapy, although efficient at transducing T cells, have considerable regulatory concerns because of their potential for insertional mutagenesis and genotoxicity (Yi *et al.*, 2005; Baum *et al.*, 2006; Nienhuis *et al.*, 2006). Also, the viral envelope proteins or viral genes expressed by some vectors can be recognized by the immune system and become a source for precipitating the immunologic destruction of transduced T cells *in vivo* (Gilbert *et al.*, 1996). The production of clinical-grade retroviral vectors and transduction of large numbers of T cells is also a laborious process that is not easily modified to rapidly screen genes of interest for their potential in adoptive immunotherapy protocols. Gene transfer methods that circumvent these problems, but can achieve meaningful advantageous modifications of T cell function *in vivo* and be readily applied clinically, are desirable for the efficient translation of gene-modified T cell therapy into humans. Furthermore, methods to enrich expanded T cell populations for antigen specificity against the desired viral or tumor antigens are desirable to enhance the potency of the infused lymphocytes and reduce potential autoimmune reactivity by nonspecific T cells.

To develop a clinical platform that simultaneously achieves both the enrichment and modification of antigen-specific T cells for use in AIT, we explored whether the observation made by us and others that RNA electroporation of T cells is greatly enhanced by prior T cell activation (Kuppen *et al.*, 2001; Van Driessche *et al.*, 2005; Zhao *et al.*, 2005; Schaft *et al.*, 2006a) could be leveraged to isolate and selectively modify the function of antigen-specific T cells responding to antigen-pulsed, autologous dendritic cells (DCs). Using green fluorescent protein (GFP) as a marker gene and the CMV antigen, pp65, for T cell stimulation, we found that greater than 95% of CMV-specific T cells could be reproducibly isolated from bulk culture through sorting of GFP-expressing T cells after electroporation of T cells that had been previously stimulated with autologous DCs pulsed with pp65 peptide or RNA. To examine the targeting of a functionally relevant protein to CMV-specific T cells, we examined the electroporation of RNA encoding CXCR2 (interleukin [IL]-8 receptor β), a C-X-C chemokine receptor (Cao *et al.*, 2000), and observed that the chemotactic function of pp65-specific T cells in response to IL-8, GRO- α (growth-related oncogene- α), and the CMV-specific chemokine UL146 could be greatly enhanced *in vitro*. Importantly, we also demonstrate that CXCR2 RNA-transfected T cells can exhibit enhanced chemotactic function *in vivo*, with preferential entry into the peritoneal cavity and the CNS in response to CXCR2-specific ligands.

These studies demonstrate that RNA electroporation represents a safe and technically simple platform that can be used to selectively modify and isolate antigen-specific T cells for use in

adoptive cellular therapy in humans and, importantly, provides *in vivo* evidence that the biologic function of T cells can be productively modified by RNA transfection.

MATERIALS AND METHODS

Dendritic cell generation, antigen loading with peptide and RNA, and maturation of dendritic cells

All cellular materials used in these experiments were obtained from normal volunteers and patients with malignant glioma after informed consent had been given, under the approval of the Duke University Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were obtained by leukapheresis and incubated for 1 hr in AIM-V medium (Invitrogen Life Technologies, Grand Island, NY) at 37°C to allow adherence to plastic and then cultured for 6 days in AIM-V medium supplemented with granulocyte-macrophage colony-stimulating factor (800 units/ml) and IL-4 (500 units/ml). Immature dendritic cells were harvested on day 6, washed, and resuspended in Opti-MEM (Invitrogen Life Technologies) at 2.5×10^7 /ml. The supernatant from DC culture was saved as conditioned medium for later use. Cells were electroporated in 2-mm cuvettes: 200 μ l of DCs (5×10^6 cells) at 300 V for 500 μ sec, using a square waveform generator (ECM 830 Electro Square Porator; BTX, a division of Genetronics, San Diego, CA). DCs were electroporated with 2.5 μ g of RNA per 10^6 cells. Cells were transferred to 60-mm tissue culture Petri dishes containing a 1:1 combination of conditioned dendritic cell growth medium and fresh medium. Cells were matured overnight with IL-1 β (5 ng/ml), tumor necrosis factor- α (5 ng/ml), IL-6 (150 ng/ml), and prostaglandin E₂ (1 μ g/ml). IL-4, tumor necrosis factor- α , IL-1 β , and IL-6 were obtained from R&D Systems (Minneapolis, MN); granulocyte-macrophage colony-stimulating factor was from the pharmacy at Duke University Medical Center (Durham, NC) and prostaglandin E₂ was from Pharmacia (Erlangen, Germany). For peptide stimulation, mature dendritic cells were washed and resuspended in 1:1 conditioned medium and fresh medium with a 10- μ g/ml concentration of the HLA-A2-restricted immunogenic human cytomegalovirus pp65 peptide NLVPMVATV (pp65₄₉₅₋₅₀₃) (AnaSpec, San Jose, CA).

Pulsing of dendritic cells with pp65 peptide or mRNA and activation of T cells

DCs were generated from HLA-A2⁺ normal volunteers and patients with malignant glioma and pulsed with pp65 peptide (10 μ g/ml) in AIM-V medium–2% human AB serum for 3 hr at 37°C. For loading with mRNA encoding pp65, DCs were washed and resuspended in Opti-MEM for electroporation. Two micrograms of mRNA encoding full-length HCMV pp65 per 1×10^6 DCs was electroporated with the BTX ECM 830. DCs were washed once with 45 ml of AIM-V medium and autologous responder lymphocytes were added at a 1:10 ratio (DC:T cells). Volumes were adjusted to 2×10^6 cells/ml and cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 3 days, an equal amount of AIM-V medium with 2% pooled human AB serum plus IL-2 (10 U/ml) was added and cells were transferred to 24-well plates at a volume of 1 ml/well. Thereafter, every 2–3 days, cells were evaluated for growth and adjusted to 1×10^6 cells/ml. After 8 to 11 days of coculture with pp65-pulsed DCs, the T cells were harvested and electroporated with mRNA encoding GFP or CXCR2.

Alternatively, T cells were stimulated by polyclonal stimulation using immobilized anti-CD3 monoclonal antibodies (Ortho Biotech, Raritan, NJ) (2 μ g/ml in phosphate-buffered saline [PBS] overnight in a T-150 flask). Cells were plated in AIM-V medium with 2% human AB serum (10^6 cells/ml) in CD3-coated plates and cultured for 3 to 5 days before harvesting.

Source of T lymphocytes

Nonadherent (NA) cells were generated from PBMCs from patients with malignant gliomas and normal volunteers under Duke University Institutional Review Board approval after informed patient consent was given. Mononuclear cells from peripheral blood were isolated by Ficoll-Hypaque gradient separation (LSM; MP Biomedicals, Solon, OH).

Electroporation of DC-pulsed pp65 peptide-stimulated T cells with GFP and CXCR2 mRNA

On the basis of the observation that only activated T cells are able to express the protein of interest, we sought to investigate the ability to select antigen-specific T cells, using mRNA as a selection marker.

The mRNA encoding GFP was prepared from the PGEM4Z/GFP/A64 vector (kindly donated by E. Gilboa, Duke University Medical Center) as previously described (Zhao *et al.*, 2003). CXCR2 mRNA was prepared from the pcDNA3.1+ vector (Missouri S&T cDNA Resource Center, Missouri University of Science and Technology, Rolla, MO) and expressed in the Psp73-sph/A64 vector (also kindly provided by E. Gilboa) (Nair *et al.*, 2003). DCs pulsed with pp65 peptide were used to stimulate autologous T cells as described above. The cells were kept in medium with 10 U/ml of IL-2 and incubated for 7 days at 37°C. For electroporation, 0.2 ml of the cell suspension (5×10^6) was mixed with 10 μ g of *in vitro*-transcribed GFP or CXCR2 mRNA. The mixtures were electroporated in 0.4-cm cuvettes (EquiBio; PEQLAB Biotechnologie, Erlangen, Germany) at 300 V and 150 Mf, using an electroporation device. After electroporation, the cells were cultured in fresh AIM-V medium supplemented with 2% human AB serum and incubated overnight. Twenty-four hours after electroporation, the cells were harvested and stained for CD8 and HLA-A2 tetramer positivity and analyzed by flow cytometry.

Expansion of sorted T cells

Using the same stimulation and electroporation methods as described above, GFP-positive and negative T cells were sorted on a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA) 24 hr posttransfection. The cells were placed in AIM-V medium supplemented with 100 U/ml of IL-2 to allow for expansion. Live cells were counted on days 0, 7, 10, 14, and 17, using light microscopy and trypan blue dye exclusion.

Cell migration assays

Activated lymphocytes (5×10^5 cells) were placed into the upper chamber of six-well filter chamber plates (Beckman Coulter, Fullerton, CA) in triplicate. Medium containing no cytokine or various concentrations of IL-8, GRO- α , or UL146 was placed into the lower chamber and cells were incubated for 45 min to 1 hr at 37°C. Medium in the lower chamber was collected, and cells were centrifuged and resuspended in 50 ml of medium and counted by trypan dye exclusion. For assays of *in vivo* migration into the peritoneal cavity, unmodified or CXCR2 RNA-transfected T lymphocytes were differentially labeled with carboxy-fluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) at 1 μ M (untransfected) and 10 μ M (CXCR2 RNA transfected) for 5 min in PBS, washed, and injected intravenously into recipient NOD/SCID mice (Taconic, Hudson, NY) at 10^7 cells per mouse. One microgram of chemokine (IL-8, UL146, or GRO- α) in 1 ml of PBS was injected intraperitoneally every 8 hr for 24 hr after injection of T cells. Mice were sacrificed, lymphocytes were harvested by peritoneal lavage with 3 ml of PBS, and cells were concentrated by centrifugation. Cells were stained with phycoerythrin (PE)-conjugated anti-human CD45 (BD Biosciences) and accumulation of untransfected (CFSE^{low}) and CXCR2 RNA-transfected (CFSE^{high}) cells was evaluated by flow cytometry. The relative accumulation of T cell populations was determined by using accumulation in PBS-treated

animals as a baseline for comparison of response to chemokine injection. For *in vivo* migration assays into the CNS, 1 μ g of UL146 in 5 μ l of PBS was injected into the right parietal lobe of mice under general anesthesia, using a stereotactic frame, and 5 μ l of PBS was injected into the left parietal lobe. CFSE (5 μ M)-labeled untransfected or CXCR2 RNA-transfected T cells were injected into recipient mice at 10^7 cells per mouse. Six hours later, mice were sacrificed and brains were harvested and dissected into right and left parietal lobes. Single-cell digests were prepared by collagenase digestion (1 mg/ml in RPMI) (Sigma-Aldrich, St. Louis, MO) of minced tissue for 30 min at 37°C. Single-cell suspensions of left and right parietal lobes were assessed for accumulation of infused lymphocytes by flow cytometric analysis of CFSE-labeled cells; 100,000 events were collected and total numbers of infiltrating T cells were determined by flow cytometric counting of CFSE-positive cells.

RESULTS

Efficiency of GFP and CXCR2 expression in anti-CD3-activated T cells

We found, as have others, that electroporation of unstimulated human PBMCs with GFP RNA did not result in expression of protein within inactivated T cells (data not shown). Stimulation of T cells with immobilized anti-CD3 antibody 48–96 hr before electroporation, however, resulted in high levels of GFP expression in 60–70% of T cells as analyzed by flow cytometry (Fig. 1). We also examined expression of the chemokine receptor CXCR2 after electroporation with CXCR2 mRNA and found CXCR2 expression in more than 80% of T cells after polyclonal stimulation and RNA electroporation (range, 60–87% in repeated experiments). Activated T cells without electroporation displayed a baseline expression of CXCR2 in 10–16% of T cells (Fig. 1).

Selection of CMV-specific CD8⁺ T cells, using RNA electroporation of GFP

The requirement for T cell activation for efficient RNA transfection could allow for the identification of antigen-specific T cells within bulk stimulated PBMC cultures. To determine the efficiency of RNA electroporation in antigen-specific T cells, PBMCs were stimulated for 7 days with autologous DCs pulsed with an HLA-A2-restricted pp65 peptide. Day 7 stimulated cultures were electroporated with GFP RNA and expression of GFP was examined in antigen-specific and nonspecific T cells, using an HLA-A2 pp65 tetramer. Forward and side scatter gating revealed activated lymphocyte cells of blastlike morphology (Fig. 2, top left). GFP expression was shown in approximately 25% of all CD8⁺ T cells (Fig. 2, top right), and tetramer analysis revealed that expression was restricted almost exclusively to pp65-specific T cells (Fig. 2, bottom right). Of T cells expressing GFP protein, 97% were identified as pp65 specific by tetramer staining. Furthermore, more than 98% of all tetramer-positive CD8⁺ T cells expressed GFP protein, indicating that RNA transfection not only was restricted to antigen-stimulated T cells (demonstrating exquisite specificity; 97.40%), but that this process also efficiently identified all of the relevant responder T cells (demonstrating high sensitivity for identifying antigen-specific T cells; 89.77% sensitivity for identifying all relevant cells). These results were confirmed in three repeat experiments, with a mean specificity for transfection of antigen-specific T cells of $93.10 \pm 5.87\%$ and sensitivity of detecting all tetramer-positive T cells by GFP expression of $86.45 \pm 8.45\%$. GFP expression in DC-stimulated T cells cultured in IL-2 (10 U/ml) was found to persist for 5–7 days (Fig. 3A), indicating the capacity to transiently express proteins in rapidly expanding T cells.

Enrichment and expansion of CMV-specific CD8⁺ and CD4⁺ T cells, using RNA electroporation of GFP

The capacity to isolate single peptide-restricted T cells can be efficiently achieved by tetramer-based cell sorting, which does not require transfection of stimulated T cells. To evaluate the capacity to identify and isolate polyclonal populations of antigen-specific CD4⁺ and CD8⁺ CMV-specific T cells, GFP RNA electroporation was conducted on T cells stimulated with DCs transfected with mRNA encoding the full-length pp65 antigen. Eleven days after *in vitro* stimulation with pp65 RNA-pulsed DCs, PBMCs from HLA-A2-positive patients ($n = 3$) were electroporated with GFP RNA and the kinetics of RNA expression were evaluated by flow cytometry (Fig. 3A). Gene expression was found to peak at 48 hr and to decline to baseline by day 7 posttransfection. Therefore, in repeat experiments T cells were electroporated on day 11 after DC stimulation and 48 hr later GFP-positive (GFP⁺) and GFP-negative (GFP⁻) T cells were separated by flow cytometric cell sorting. Sorted cells were analyzed by tetramer analysis and cytokine flow cytometry for enrichment of CMV-specific T cells. Equal numbers of GFP⁺ and GFP⁻ cells were placed back into culture containing IL-2 (100 U/ml) and expansion was evaluated on days 7, 10, 14, and 17 postsorting. Interestingly, only GFP⁺ T cells were capable of further expansion when cultured with IL-2, indicating that RNA electroporation can efficiently separate T cells capable of further expansion *in vitro* from anergic or inactivated T cells poststimulation with RNA-pulsed DCs (Fig. 3B). To determine whether GFP expression was simply separating proliferating T cells from anergic cells, GFP⁺ and GFP⁻ sorted cells were also stimulated with a polyclonal expansion platform consisting of allogeneic feeder cells (PBMCs from an unmatched normal donor), anti-CD3, and high-dose IL-2 (100 U/ml) in a rapid expansion protocol (REP) suitable for generating clinical-scale T cell expansions within 14 days (>1000-fold expansion) (Dudley *et al.*, 2003). Using the REP protocol, both GFP⁻ and GFP⁺ T cells were expanded efficiently, demonstrating that the capacity for RNA transfection does not segregate functional from nonfunctional T cells, but rather identifies T cells that have received sufficient activation during the coculture with antigen-pulsed dendritic cells to enter mitotic cell cycling.

One of the three patient samples examined demonstrated expansion of both HLA-A2- and HLA-B7-restricted pp65-specific CD8⁺ T cells after stimulation with RNA-pulsed DCs. After RNA electroporation and sorting, the GFP⁺ fraction contained 95% of the HLA-A2-restricted T cells and 98% of the HLA-B7-restricted T cells, representing a 20- to 50-fold enrichment of antigen-specific T cells within the GFP⁺ fraction, and demonstrating efficient identification and separation of almost all of the antigen-specific CD8⁺ T cells (Fig. 3C). Similar results were obtained with two other patients who demonstrated only HLA-A2-restricted pp65 T cell reactivity. To determine the capacity to enrich for CMV-specific CD4⁺ T cells, cytokine flow cytometry was performed with unstimulated, *Staphylococcus enterotoxin B* (SEB)-stimulated, or CMV pp65 peptide mix-stimulated T cell fractions. The positive control (SEB-stimulated T cells) indicated that 13.69% of the CD4⁺ T cells secreted IFN- γ in the GFP-negative fraction and 22.49% of the GFP⁺ CD4⁺ T cells secreted IFN- γ , confirming that both fractions contained functional effector CD4⁺ T cells capable of responding to superantigen. However, IFN- γ secretion in response to the CMV peptide mix by CD4⁺ T cells was restricted to the GFP⁺ fraction, with 14.15% of CD4⁺ T cells secreting cytokine compared with 0.31% of CD4⁺ T cells in the GFP⁻ fraction (Fig. 3D). These results demonstrate a 45-fold enrichment of antigen-specific CD4⁺ T cells within the GFP⁺ fraction after separation of T cells stimulated with pp65-pulsed DCs and demonstrate that RNA electroporation is an efficient means of identifying antigen-specific CD8⁺ and CD4⁺ T cells within bulk PBMC cultures for enrichment and further evaluation. Furthermore, the capacity to identify and enrich polyclonal populations of antigen-specific CD8⁺ and CD4⁺ T cells, using RNA transfection, provides a simple method for generating populations of T

cells with broad antigen recognition and effector function for use in adoptive immunotherapy.

Expression of CXCR2 mRNA in antigen-specific T cells

Having demonstrated the capacity to selectively express marker genes in antigen-specific T cells using RNA electroporation, we sought to determine whether the functional properties of CMV-specific T cells could be modulated by RNA transfection. We examined the capacity to modulate the migratory function of T cells by expression of the chemokine receptor CXCR2, after CXCR2 RNA electroporation into T cells stimulated with pp65-pulsed DCs. CXCR2 expression was monitored in tetramer-positive versus tetramer-negative CD8⁺ T cells, using CXCR2-specific antibodies and flow cytometry. We found that 10–15% of stimulated CD8⁺ T cells expressed CXCR2 on their surface (Fig. 1) at baseline and after CXCR2 RNA transfection approximately 50% of CMV-specific T cells expressed CXCR2 on their surface (Fig. 4). Tetramer-negative T cells, in contrast, did not exhibit CXCR2 expression above background (10.9%), indicating selective expression of the chemokine receptor in antigen-specific T cells after RNA electroporation.

Enhanced chemotactic function of CXCR2 RNA-modified T cells *in vitro*

To examine the capacity to confer enhanced function to RNA-modified T cells, we evaluated the chemotactic function of unmodified, GFP RNA-transfected, and CXCR2 RNA-transfected T cells in Transwell migration assays in response to the CXCR2-specific ligands IL-8, GRO- α , and UL146 (Fig. 5A–C). CXCR2 RNA-transfected T cells exhibited a dose-responsive enhanced chemotactic response to all three ligands compared with unmodified or GFP RNA-transfected T cells. Most notably, the chemotactic response toward IL-8, secreted at high levels within a number of tumors, and UL146, a chemokine produced by human cytomegalovirus, and the only ligand exclusive to CXCR2, were increased more than 300–500% in CXCR2-modified T cells.

Enhanced migration of CXCR2 RNA-modified T cells *in vivo*

To determine whether transfection of CXCR2 RNA into activated T cells could facilitate enhanced T cell migration *in vivo*, we evaluated the migration of RNA-modified T cells into the peritoneal cavity and CNS of SCID mice that had been injected with CXCR2-specific chemokines. Mice were injected intraperitoneally with chemokines (1 μ g/ml) every 8 hr for 24 hr postinfusion of differentially CFSE-labeled untransfected or CXCR2 RNA-transfected T cells. Mice were sacrificed and cells were harvested by peritoneal lavage, and the accumulation of CXCR2-transfected and untransfected T cells was analyzed by flow cytometry by gating on human CD45⁺ cells and evaluating the relative accumulation of CFSE^{low} cells (un-transfected) to CFSE^{high} cells (CXCR2 RNA transfected). The relative accumulation of T cells in chemokine-injected mice was compared with that of PBS-injected mice and is shown in Fig. 6A. Results demonstrate the accumulation of CXCR2 RNA-transfected cells in response to UL146 and IL-8. The greatest chemotactic response was to UL146, which paralleled findings of our *in vitro* studies. No significant accumulation was observed in response to GRO- α in this *in vivo* assay. We also examined whether CXCR2 RNA modification could lead to accumulation of T cells within the CNS in response to UL146. Immunodeficient mice were administered a single injection of PBS in the left frontal parietal lobe, or of UL146 (1 μ g) in the right frontal parietal lobe, under guidance of a stereotactic frame. CFSE-labeled untransfected or CXCR2 RNA-transfected T cells were injected intravenously and 6 hr postinjection, left and right cerebral hemispheres were harvested, single-cell digests were prepared, and detection of CFSE-labeled T cells was evaluated by flow cytometry. As shown in Fig. 6B, CXCR2 RNA-modified T cells exhibited enhanced infiltration of the UL146-injected hemispheres compared with PBS-injected hemispheres, whereas untransfected T cells exhibited no significant difference in infiltration

of either hemisphere. Preferential accumulation of CXCR2 RNA-modified T cells at later time points was not observed, likely secondary to the transient gradient of chemokine established by the intracranial injection. These results demonstrate that CXCR2 RNA-modified T cells exhibit enhanced chemotactic function *in vitro* and *in vivo* and that RNA-based modification of T cells can be used to selectively identify and modify antigen-specific T cells.

DISCUSSION

Clinical studies by Dudley and colleagues (2002, 2005) using the adoptive transfer of tumor-specific lymphocytes in lymphodepleted patients have highlighted the efficacy of AIT in the treatment of refractory malignant disease. These studies have demonstrated a correlation between clinical responsiveness to AIT and the magnitude and duration of antigen-specific T cells that are achieved in the circulation of treated patients. For effective cellular immunotherapy against viruses and tumors, the generation of large numbers of functional antigen-specific T cells *in vitro* for use in AIT is a desirable goal. Methods to enrich antigen-specific T cells and modify their function, using gene transfer technology, have not been readily applied clinically, in part due to the cumbersome and elaborate protocols required to generate such T cell populations.

We explored a safe and simplified gene transfer technology employing the electroporation of messenger RNA encoding genes that could be used to identify and separate transfected from untransfected T cell populations, as well as modify their migratory function. Using GFP as a model marker gene, we demonstrate that CMV-specific T cells can be readily identified and separated from cultures stimulated with pp65-pulsed DCs, and that RNA transfection can be used to simultaneously isolate both antigen-specific CD4⁺ and CD8⁺ T cells. The capacity to enrich for antigen-specific CD4⁺ T cells is of particular utility, as MHC class II-restricted tetramer isolation techniques have not been described for the majority of viral or tumor antigens. It is unclear at this time what mechanisms govern efficient protein expression after RNA electroporation preferentially directed to activated T cells (Van Tendeloo *et al.*, 2001; Schaft *et al.*, 2006b), but we have demonstrated in our study the capacity to leverage this observation to identify antigen-specific T cells stimulated with autologous DCs with high sensitivity and specificity. Other methods of isolating antigen-specific T cells have been reported, such as retroviral gene marking of proliferating T cells, tetramer isolation of peptide-specific T cells, and IFN- γ cytokine capture assays, each of which has particular advantages and disadvantages relative to their use in large-scale clinical applications. RNA-mediated transfection of antigen-specific T cells may have particular advantages in being able to simultaneously enrich the purity and modify the function of antigen-specific T cells by a single strategy, such as the electroporation of chemokine receptor or other surface receptors of interest and isolation of cells on the basis of the expression of the transfected receptor. The expression of genes encoding proteins that significantly modulate the function of T cells, such as CXCR2, can be efficiently targeted preferentially to those T cells with a specificity of interest, using an antigen-specific stimulation platform followed by RNA electroporation. Similar data have been demonstrated by retroviral transduction of proliferating T cells in response to antigen-specific T cell stimulation; however, the generation of clinical-grade retroviral constructs for use in AIT is technically more challenging than RNA generation and is not without considerable safety concerns.

The transient nature of gene expression by RNA electroporation will limit the expression of functional genes to those whose expression for only a few days is expected to convey a significant therapeutic advantage *in vivo*. Genes that enhance the migration of T cells to sites of inflammation, viral infection, or tumor progression, such as chemokine receptors,

represent a class of proteins whose transient expression might significantly enhance the efficacy of adoptively transferred lymphocytes. CXCR2 is normally expressed only in a small fraction of T lymphocytes and mediates chemotaxis toward IL-8, GRO- α , and UL146, the latter a CMV-specific chemokine (Murphy, 1997; Robertson, 2002; Reutershan, 2006). IL-8 has been shown to be increased in sites of inflammation as well as dramatically elevated within gliomas and other tumors (Van Meir *et al.*, 1992; Salmaggi *et al.*, 2003). GRO- α has been shown to be increased in expression in a number of malignancies including melanoma (Kershaw *et al.*, 2002), gastrointestinal cancers (Cuenca *et al.*, 1992; Li *et al.*, 2004), and malignant gliomas (Zhou *et al.*, 2005). Its expression in gliomas has been shown to correlate with tumor grade (Zhou *et al.*, 2005), rendering T cells with chemotactic function toward GRO- α , a potentially useful effector in tracking down invasive high-grade lesions. UL146 is a chemokine synthesized by human cytomegalovirus with exquisite specificity for CXCR2 (Saederup and Mocarski, 2002; Hassan-Walker *et al.*, 2004). Although the role of UL146 in viral infection is unclear, the localization of CMV-specific T cells expressing CXCR2 to sites of viral infection through UL146-mediated chemoattraction could potentially enhance the efficacy of AIT in the treatment of active viral infection or early reactivation.

The capacity to modify T cell function *in vitro* by RNA electroporation has been previously reported (Van Tendeloo *et al.*, 2001; Schaft *et al.*, 2006b; Zhao *et al.*, 2006b), and we demonstrate, for the first time, the *in vivo* functional use of RNA-modified T cells in this study: enhanced trafficking of CXCR2 RNA-modified T cells into the peritoneal cavity in response to IL-8 and UL146 and into the CNS of NOD/SCID mice in response to UL146, the latter demonstrating the rapid accumulation of CXCR2-modified T cells within the brain. These studies demonstrate the potential to enhance T cell localization to sites of therapeutic interest, using RNAs encoding chemokine receptors or other chemotactic receptors. This may be of particular use in the targeting of brain tumors, as the CNS displays significantly limited normal trafficking of circulating immune cells (Oldstone and Southern, 1993; Hickey, 1999; Li *et al.*, 2005). This approach may also have significant potential to direct antigen-specific effector T cells to sites of tumor growth, viral infection, or vaccine sites for preferential expansion. Alternatively, this approach can be used to direct regulatory T cell populations to sites of immunopathology or autoimmune inflammatory disease where attenuation of ongoing immune responses may be desirable. The capacity to limit gene expression to T cells stimulated against antigens of interest allows for specific modification of antigen-specific T cells while leaving nonspecific lymphocytes unmodified. This specificity can be used to enrich for or deplete target T cell populations such as virus-specific and alloreactive T cells, respectively, allowing for increased effectiveness of the expanded T cell population and potential reduction in the likelihood of autoimmune pathology. Our laboratory is currently exploring the potential to direct the trafficking of tumor-specific T cells to sites of tumor growth in patients with high-grade malignant gliomas using RNA-modified T cells.

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FIG. 1.

High-efficiency gene expression in human T cells, using RNA electroporation. Nonadherent cells from PBMCs were stimulated *in vitro*, using anti-CD3-coated plates, for 72 hr and harvested, washed, and electroporated with RNA encoding GFP or CXCR2 (2 μ g of RNA per 10⁶ cells) as described in Materials and Methods. Cells were cultured in medium (AIM-V medium, 2% AB serum, hIL-2 [100 U/ml]) for 48 hr, washed, and analyzed for GFP expression and CXCR2 by administration of allophycocyanin (APC)-conjugated CXCR2-specific monoclonal antibody and flow cytometry. *Top*: Untransfected T cell expression of GFP (*top left*) and CXCR2 (*top right*). *Bottom*: GFP expression after electroporation with GFP RNA (*bottom left*) and CXCR2 expression after electroporation with CXCR2 RNA (*bottom right*). This experiment was repeated twice with similar results.

FIG. 2.

RNA expression is restricted to antigen-specific T cells stimulated by autologous DCs. PBMCs from an HLA-A2-positive donor were stimulated for 7 days with autologous DCs pulsed with an HLA-A2-restricted pp65 peptide (N9V), harvested, and electroporated with GFP RNA. Forty-eight hours later GFP expression was examined in N9V-tetramer-positive versus N9V-tetramer-negative CD8⁺ T cells. *Left:* Gating on blastlike lymphocytes (R1; *top*) and CD8⁺ T cells (R2; *bottom*). *Middle:* No GFP expression in cells transfected with a control RNA (CXCR2). *Right:* GFP expression in 24.57% of CD8⁺ T cells (*top right*), which consists almost entirely of pp65-specific T cells as shown by tetramer staining (gated on lymphocytes (R1) and CD8⁺ cells (R2)) (*bottom right*).

FIG. 3.

(A) Kinetics of GFP expression in RNA-transfected T cells stimulated with DCs. PBMCs were stimulated for 11 days *in vitro* with autologous DCs pulsed with pp65 mRNA as described in Materials and Methods. Cells were harvested and transfected with GFP RNA ($2 \mu\text{g}/10^6$ cells). Expression of GFP in tetramer-positive (HLA-A2⁺) and tetramer-negative cells was evaluated beginning 24 hr after electroporation and monitored until day 7 postelectroporation. The percentage of GFP-expressing cells is shown. (B) Expansion of sorted GFP RNA-transfected T cells. PBMCs were stimulated for 11 days *in vitro* with autologous DCs pulsed with pp65 mRNA as described in Materials and Methods. Cells were harvested and transfected with GFP RNA ($2 \mu\text{g}/10^6$ cells). Forty-eight hours later, cells were sorted by flow cytometry on the basis of GFP expression (GFP⁺ and GFP⁻) and placed back into culture with high-dose IL-2. Expansion of the cells was evaluated by trypan blue staining and counting of an aliquot of cells every 3–4 days. This experiment was repeated with PBMCs from another donor with similar results. (C) Tetramer analysis of HLA-A2- and HLA-B7-restricted pp65-specific T cells. A panel of tetramers was used to identify patients with more than one type of haplotype-restricted T cell reactivity against pp65 detectable by tetramer. *Left*: Frequency of HLA-A2- and HLA-B7 pp65-specific T cells in the GFP⁻ fraction. *Right*: Frequency of tetramer-positive cells in the GFP⁺ fraction. (D) Isolation of CMV-specific CD4⁺ T cells by RNA electroporation. PBMCs stimulated with pp65 RNA-pulsed DCs were generated as previously described and electroporated with GFP RNA on day 11 after DC stimulation. Cells were sorted into GFP⁺ and GFP⁻ fractions and 48 hr later were analyzed by intracellular cytokine flow cytometry for IFN- γ production after exposure to no antigen, SEB ($1 \mu\text{g}/\text{ml}$), or CMV pp65 peptide mix (Beckman Coulter). *Left*: Expression of IFN- γ in GFP⁻ CD4⁺ T cells. *Right*: IFN- γ in GFP⁺ CD4⁺ T cells.

FIG. 4.

Selective expression of CXCR2 in pp65-specific T cells after RNA electroporation. PBMCs were stimulated for 11 days *in vitro* with autologous DCs pulsed with pp65 mRNA as described in Materials and Methods. Cells were harvested and transfected with CXCR2 RNA (2 μ g/10⁶ cells). Forty-eight hours later, cells were harvested and analyzed for CXCR2 expression in tetramer-positive (HLA-A2-restricted donor) and tetramer-negative CD8⁺ cells. Baseline expression in activated CD8⁺ T cells of CXCR2 was approximately 10% (data not shown). Results demonstrated that RNA electroporation resulted in increased CXCR2 expression in CMV-specific T cells only, with expression in 49.1% of tetramer-positive cells, whereas no increase in expression over baseline was observed in tetramer-negative cells after DC stimulation.

FIG. 5.

(A) *In vitro* chemotaxis of CXCR2 and GFP RNA-transfected T cells toward IL-8. PBMCs were activated *in vitro* using immobilized anti-CD3 antibody-mediated stimulation for 4 days as described in Materials and Methods. Cells were harvested and transfected with CXCR2 or GFP RNA ($2 \mu\text{g}/10^6$ cells). Forty-eight hours later, cells were harvested, counted, and placed in triplicate into filter chamber culture plates as described in Materials and Methods. The migration of untransfected, GFP RNA-transfected, and CXCR2 RNA-transfected T cells in response to increasing concentrations of IL-8 was assessed after 45 min of culture in the presence of the indicated concentration of IL-8 in the lower chamber. Results demonstrated enhanced chemotactic response of CXCR2 RNA-transfected T cells compared with untransfected and GFP RNA-transfected cells ($*p < 0.05$; *t* test). This experiment has been repeated several times with the same results. (B) *In vitro* chemo-taxis

of CXCR2 and GFP RNA-transfected T cells toward GRO- α . Cells were assayed as described above against increasing concentrations of GRO- α in the lower chamber of the filter chamber plates. CXCR2-transfected T cells exhibited significantly increased chemotactic activity in response to GRO- α , whereas GFP RNA-transfected and untransfected T cells showed no significant change in migration in response to GRO- α (* p < 0.05; t test). (C) *In vitro* chemotaxis of CXCR2 and GFP RNA-transfected T cells toward UL146. Cells were assayed as described above against increasing concentrations of the CMV-secreted chemokine UL146 in the lower chamber of the filter chamber plates. CXCR2-transfected T cells exhibited markedly increased chemotactic activity in response to UL146, whereas GFP RNA-transfected and untransfected T cells showed no change in migration in response to UL146 (** p < 0.01; t test).

FIG. 6.

(A) Migration of CXCR2 RNA-transfected T cells into the peritoneal cavity. PBMCs were stimulated for 11 days *in vitro* with autologous DCs pulsed with pp65 mRNA as described in Materials and Methods. Cells were harvested and transfected with CXCR2 RNA (2 μ g/10⁶ cells). Forty-eight hours later, untransfected and CXCR2 RNA-transfected cells were harvested and differentially labeled with CFSE (1 μ M for untransfected cells and 10 μ M for CXCR2 RNA-transfected cells) for 5 min in PBS, washed, mixed, and injected intravenously into SCID mice (2 $\times$ 10⁷ cells per mouse, *n* = 3 per group). Mice were injected intraperitoneally with PBS only or with chemokines (GRO-a, IL-8, or UL146) at 1 μ g/ml in PBS every 8 hr for 24 hr after injection of T cells. Lymphocytes were harvested after sacrifice by peritoneal lavage and centrifuged, and the relative accumulation of untransfected and CXCR2 RNA-transfected cells was evaluated by flow cytometric analysis. Accumulation of CFSE^{high} cells (CXCR2 RNA-transfected T cells) and CFSE^{low} cells (untransfected T cells) in PBS-treated animals was used as a baseline for comparison with the accumulation of cells in cytokine-treated animals. Data are displayed as fold change compared with PBS-treated animals. (B) Migration of CXCR2 RNA-transfected T cells into the CNS. PBMCs were stimulated for 11 days *in vitro* with autologous DCs pulsed with pp65 mRNA as described in Materials and Methods. Cells were harvested and transfected with CXCR2 RNA (2 μ g/10⁶ cells). Forty-eight hours later, un-transfected and CXCR2

RNA-transfected cells were harvested and labeled with CFSE ($5 \mu M$) for 5 min in PBS, washed, and injected intravenously into SCID mice (1×10^7 cells per mouse, $n = 3$ per group). Anesthetized mice were subsequently injected via the right parietal lobe with the CMV chemokine UL146 ($1 \mu g$ in $5 \mu l$ of PBS) and via the left parietal lobe with PBS only. The accumulation of CFSE-labeled cells in the right and left parietal lobes was assessed 6 hr later by flow cytometry after dissection of the cerebral hemispheres and single-cell digest preparation from the parietal lobes. A representative flow cytometric dot plot of a mouse injected with CXCR2 RNA-transfected T cells shows greater accumulation of lymphocytes within the right parietal lobe injected with the CXCR2 ligand UL146. (C) *In vivo* migration of CXCR2-transfected T cells. The accumulation of T cells within the right (UL146-injected) and left (PBS-injected) parietal lobes was assessed by flow cytometry and human T cells per 100,000 events were plotted. Results demonstrated significant accumulation of CXCR2 RNA-transfected T cells within the CNS in response to UL146, whereas untransfected T cells did not exhibit any preferential accumulation ($*p < 0.05$; t test).