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J Immunol 2003; 170:5892-5896; doi: 10.4049/jimmunol.170.12.5892

http://www.jimmunol.org/content/170/12/5892
Transfection of Dendritic Cells with RNA Induces CD4- and CD8-Mediated T Cell Immunity Against Breast Carcinomas and Reveals the Immunodominance of Presented T Cell Epitopes

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Transfection of dendritic cells (DC) with tumor-derived RNA has recently been shown to elicit tumor-specific CTL capable of recognizing and lysing a variety of tumor cells. In our study we analyzed the induction of HLA class I- and II-restricted T cell responses against MCF-7 breast cancer cells. Using this approach we were able to elicit CD4- and CD8-mediated antitumor responses. The CTL specifically lysed MCF-7 cells and DC electroporated with MCF-7 RNA, but spared control cell lines. The specificity of the cytotoxic activity was confirmed in cold target inhibition assays and using mAbs blocking HLA class I molecules. Interestingly, these polyclonal cytotoxic T cells recognized selectively two epitopes derived from the MUC1 and Her-2/neu tumor Ags. The induced Th cells were found to be entirely HLA class II restricted and showed a significant cross-reactivity to a renal cell carcinoma cell line, similar to the results obtained with cytotoxic T cells.


The development of protocols for the ex vivo generation of dendritic cells (DC)¶ (1–9), which are recognized as the most powerful APC able to induce and maintain primary immune responses (10–17), led to the design of a vast array of potential vaccination strategies for the treatment of malignant and infectious diseases. One strategy that has been applied in most clinical trials is the pulsing of DC with synthetic peptides derived from tumor-associated Ags (TAA) (18, 19). This approach is limited, however, to patients who express a certain HLA haplotype and necessitates characterization of the targeted tumor Ag. Furthermore, it ignores the important role of HLA class II-restricted Th cells in initiating and maintaining an effective immune response. Therefore, alternative whole tumor strategies were developed by using DC loaded with full-length recombinant proteins, dying tumor cells (apoptotic bodies, necrotic cells), DC fused with tumor cells (20–24), and gene-based delivery of TAA into DC (25–28).

Recently, it was shown that DC transfected with mRNA coding for a certain TAA or with total tumor RNA are able to elicit potent Ag- and tumor-specific CTL responses against colorectal cancer, prostate cancer, or renal cell carcinoma (29–32). This polyvalent vaccine is able to induce T cell responses against multiple naturally processed and presented immunodominant epitopes and may thus reduce the occurrence of clonal tumor escape phenomena. Additional targeting of HLA class II-restricted epitopes might further amplify and prolong the induced T cell responses. In contrast to other whole tumor vaccine approaches using tumor cell lysates, hybrids of DC with tumors, or dead cells that are limited by the requirement for large amounts of tumor material, RNA-transfected DC could be applied even in patients with small tumors, as it was demonstrated that RNA amplified from a few tumor cells can be used for DC transfections and can be very effective in stimulating antitumor immunity (33, 34).

In our study we show that DC transfected with total tumor RNA are able to induce potent CTL responses against breast cancer cells, thus indicating that this method of Ag delivery may be useful to stimulate immune responses even against less immunogenic malignancies. Moreover, we show that the in vitro-induced CTL specific for the breast cancer cell line MCF-7 cross-reacted with a renal cell carcinoma cell line and selectively recognized two peptide epitopes derived from the MUC-1 and Her-2/neu tumor Ags, but not other immunogenic epitopes derived from the same Ags. These results are of particular interest because they demonstrate that polyclonal CTL responses stimulated by whole cell-derived Ags might have a selective specificity for a subset of presented peptides and do not recognize all epitopes expressed in the tumor cells. Furthermore, we were able to induce breast cancer-specific CD4+ Th cells using DC transfected with total tumor RNA, indicating that this technique is able to elicit immune responses directed against HLA class I and class II epitopes.

Materials and Methods

Tumor cell lines

Tumor cell lines used in the experiments were grown in RPMI 1640 medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics). The following HLA-A2-expressing tumor cell lines were used: MCF-7 (breast cancer) and A498 (renal cell carcinoma). Croft (HLA-A2) is an EBV-immortalized B cell line and was provided by O. J. Finn (Pittsburgh, PA). SK-OV-3 (HLA-A3) is an ovarian carcinoma cell line. ACHN (HLA-A26) is derived from renal cell carcinoma.

Abbreviations used in this paper: DC, dendritic cell; EGF, enhanced green fluorescent protein; RCC, renal cell carcinoma; TAA, tumor-associated Ag.
Cell isolation and generation of DC from adherent PBMC

Generation of DC from PBMC was performed as described previously (6, 18, 19). In brief, PBMC were isolated by Ficoll/Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation of heparinized blood obtained from buffy coat preparations of healthy volunteers from the blood bank of University of Tübingen. Cells were seeded (1 × 10^6 cells/ml) into six-well plates (Corning, Cambridge, MA) in RPMI 100 medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics). After 2 h of incubation at 37°C, nonadherent T cells were removed, and the adherent blood monocytes were cultured in RPMI 100 medium supplemented with the following cytokines: human recombinant GM-CSF (100 ng/ml; Leukomax; Novartis, Nutley, NJ) and IL-4 (1000 U/ml; Genzyme, Cambridge, MA) for the generation of immature DC. For maturation, DC were additionally cultured with TNF-α (1000 IU/ml; Genzyme, Cambridge, MA) for 24 h after day 6. The phenotype of DC was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Heidelberg, Germany) after 7 days of culture.

Immunostaining

Cell staining was performed using FITC- or PE-conjugated mouse mAbs against CD86, CD40 (BD PharMingen, Hamburg, Germany), CD80, HLA-DR, CD14, CD54, CD4, CD8 (BD Biosciences), CD83 (Coulter-Immunotech, Hamburg, Germany), and CD1a (OKT6; Ortho Diagnostic Systems, Seattle, WA). Appropriate mouse IgG isotypes were used as controls (BD Biosciences). The samples were analyzed by flow cytometry.

RNA isolation

Total RNA was isolated from tumor cell lysates using RNasey Maxi anion exchange spin columns (Qiagen, Hilden, Germany) according to the protocol for isolation of total RNA from animal cells provided by the manufacturer. The quantity and purity of RNA were determined by UV spectrophotometry. RNA samples were routinely checked by formaldehyde/agarose gel electrophoresis for size and integrity and were stored at −80°C in small aliquots.

Electroporation of DC

Before electroporation on day 6, immature DC were washed twice with serum-free X-VIVO 20 medium (BioWhittaker, Walkersville, MD) and resuspended to a final concentration of 2 × 10^7 cells/ml. Subsequently, 200 μl of the cell suspension was mixed with 10 μg of total tumor RNA and electroporated in a 4-mm cuvette using an Easyject Plus unit (Peqlab, Erlangen, Germany). The physical parameters were: voltage of 300 V, capacitance of 150 μF, resistance of 1540 Ω, and pulse time of 231 ms. After electroporation the cells were immediately transferred into RPMI 100 medium and returned to the incubator. The viability of the cells was >80% after electroporation, as assessed by propidium iodide staining (35).

Induction of tumor-specific CTL using DC transfected with tumor RNA

DC were transfected with MCF-7 RNA using electroporation as described above. After transfection, DC were incubated for 24 h in RPMI 100 medium containing 10 ng/ml TNF-α for maturation of DC. For CTL induction, 5 × 10^5 DC (transfected with tumor RNA) were incubated with 2.5 × 10^6 autologous PBMC in RPMI 10 medium. Additional DC transfected with RNA were stored at −80°C to be used for restimulation. After 7 days of culture, cells were restimulated with autologous RNA-transfected DC, and 1 ng/ml human rIL-2 (Genzyme) was added on days 1, 3, and 5. The cytolytic activity of induced DC was analyzed on day 5 after restimulation in a standard 51Cr release assay.

CTL assay

The standard 51Cr-labeled release assay was performed as previously described (19). Target cells were pulsed with 50 μg/ml peptide for 2 h (for peptide-pulsed target cells) and labeled with 51Cr sodium chromate in RPMI 100 medium for 1 h at 37°C. Cells (10^4) were transferred to a well of a round-bottom, 96-well plate. Varying numbers of CTL were added to give a final volume of 200 μl, and cells were incubated for 4 h at 37°C. At the end of the incubation, supernatants (50 μl/well) were harvested and counted in a β-plate counter. The percentage of specific lysis was calculated as: 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release). Spontaneous and maximal releases were determined in the presence of either RPMI 100 medium or 1% Triton X-100, respectively. The Ag specificity of tumor cell lysis was further assessed in a cold target inhibition assay by analyzing the capacity of cold (unlabeled) MCF-7 cells to block lysis of hot (labeled) MCF-7 cells and by blocking HLA class I molecules using an mAb (W06/32, 20 μg/ml; provided by S. Stevanovic, Tubingen, Germany).

Induction of tumor-specific Th cells (CD4+) using DC transfected with MCF-7 RNA

CD4+ T lymphocytes were isolated from PBMC using the CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD4+ T cells was assessed after isolation by flow cytometry. DC were transfected with MCF-7 total RNA by electroporation on day 6 as described above. After transfection, DC were incubated for 24 h in RPMI 100 medium containing 10 ng/ml TNF-α for maturation of DC. For Th cell induction, 2.5 × 10^5 CD4+ T lymphocytes were cocultured with 5 × 10^6 autologous DC (transfected with MCF-7 tumor RNA). On days 7 and 14 after Th cell induction, restimulations were performed using 5 × 10^5 autologous DC (transfected with MCF-7 tumor RNA) each time. IL-2 was added every second day following the first restimulation (2 ng/ml). The Ag specificity of the induced CD4+-mediated immune response was assessed on day 20 after T cell induction in a [3H]thymidine proliferation assay as described below.

Proliferation assay

A total of 2 × 10^5 responding cells (CD4+ T lymphocytes) were cultured in 96-well, flat-bottom microplates (Nunc, Wiesbaden, Germany) with 10^6 DC. Stimulation with PMA/ionomycin was used as a positive control, whereas unstimulated CD4+ T lymphocytes and stimulation with DC electroporated with irrelevant enhanced green fluorescence protein (EGFP) (35) RNA served as negative controls. Inhibition of HLA class I or class II peptide-pulsed target cells) and labeled with 51Cr sodium chromate in RPMI 100 medium. Additional DC transfected with RNA were stored at −80°C to be used for restimulation. After 7 days of culture, cells were restimulated with autologous RNA-transfected DC, and 1 ng/ml human rIL-2 (Genzyme) was added on days 1, 3, and 5. The cytolytic activity of induced DC was analyzed on day 5 after restimulation in a standard 51Cr release assay.

Induction of MCF-7-specific CTL using RNA-transfected DC

In our study we analyzed the feasibility of total tumor RNA transfection into DC as a means to induce CD8+-mediated T cell responses using the breast cancer cell line MCF-7, which is regarded to be less immunogenic. As shown in Fig. 1A, transfection of DC generated from an HLA-2-positive healthy donor with tumor RNA isolated from MCF-7 cells resulted in the induction of CTL that significantly recognized MCF-7 cells and autologous DC electroporated with MCF-7-RNA, but not control cell lines or untreated DC in a standard 51Cr release assay. The lysis of MCF-7 cells could be blocked by an mAb directed against HLA class I molecules, demonstrating that the cytolytic activity of the in vitro-induced CTL was HLA class I restricted (Fig. 1B). To further analyze the specificity of these CTL, we performed cold target inhibition assays. As shown in Fig. 1B, the lysis of MCF-7 cells (pulsed with 51Cr, hot targets) could be blocked by addition of unpulsed MCF-7 cells (cold targets), but not by SK-OV-3 tumor cells. Interestingly, we also observed that CTL induced using DC transfected with MCF-7 RNA efficiently recognized A498 renal cell carcinoma cells, but spared other control cell lines, thus suggesting that tumor Ags might be shared among these cell lines (Fig. 2A).
Epitopes derived from MUC1 and Her-2/neu tumor Ags contribute to the cytotoxic activity of MCF-7-specific CTL

We have recently demonstrated that breast cancer and RCC tumor cells present HLA-A2-restricted T cell epitopes derived from MUC1 (peptides M1.1 and M1.2) and Her-2/neu (peptides E75 and GP2) and can be lysed by CTL specific for these peptides (18, 36). To further analyze the specificity of the in vitro-induced CTL, we included target cells pulsed with the antigenic MUC1 (M1.1 and M1.2) or Her-2/neu (E75 and GP-2) peptides or with an irrelevant HIV peptide. As shown in Fig. 2B, the MCF-7-specific CTL recognized target cells pulsed with the M1.2 and E75 peptides. On the other hand, they did not lyse cells pulsed with the other two peptides derived from these Ags, suggesting that the M1.2 and E75 peptides might represent immunodominant epitopes.

Induction of CD4+ Th lymphocytes using MCF-7 transfected DC

In the next set of experiments we analyzed the induction of a CD4-mediated Th cell response by RNA-transfected DC. CD4+ T lymphocytes were isolated using magnetic bead technology (the purity of CD4+ T cells was routinely found to be >90% as assessed by flow cytometry) and were stimulated with autologous DC transfected with MCF-7 total RNA. As demonstrated in Fig. 3, [3H]thymidine proliferation assays performed after two restimulations revealed a tumor-specific proliferation pattern with a cross-reactivity to A498 cells. DC transfected with MCF-7 or A498 RNA induced significant proliferation, whereas DC transfected with irrelevant RNA did not. The induced proliferative response could be blocked using an mAb directed against HLA class II molecules, but not with an mAb against HLA class I molecules, thus demonstrating that the induced T cell population was entirely HLA class II restricted.

Our study shows that RNA transfection of DC is a feasible approach to induce HLA class I- and HLA class II-restricted immune responses, even in less immunogenic malignancies such as breast carcinoma. Furthermore, these data give evidence that polyclonal CTL responses induced by total tumor RNA-transfected DC might exhibit a selective specificity for certain epitopes derived from shared tumor Ags. These results are relevant, since they demonstrate that polyclonal CTL responses stimulated by whole cell-derived Ags probably do not recognize all epitopes expressed in the tumor cells, but are directed against a certain subpopulation of peptides, which may be preferentially processed and presented by APC. This might reflect a competition among the antigenic peptides during Ag processing and presentation, and differences in the ability of the generated peptides to bind and stabilize the MHC complex. In this regard the MUC1-derived M1.2 peptide shows a higher binding affinity to the HLA-A2 molecule compared with the M1.1 peptide (P. Brossart, unpublished observations). Furthermore, the M1.2 peptide is deduced from the signal sequence of the MUC1 protein and therefore might be presented independently of the TAP molecule, which could be of advantage during Ag presentation (37). The GP2 peptide was recently shown to have a reduced binding affinity to the HLA-A2 molecule due to a lack of stabilizing contacts with the peptide binding cleft (38, 39). Interestingly, we have previously demonstrated that the M1.2 and E75 peptides elicited a stronger CTL response in vivo compared with the M1.1 and GP2 peptides when these four epitopes were applied in a clinical trial using peptide-pulsed DC (19). Another possible explanation for the differential activation of CTL could be represented by the differences in the Ag processing machinery of DC and tumor cells. In this context, it was recently shown that DC and
bated with 10^5 DC for 5 days. The induced proliferation was determined on DC electroporated with EGFP RNA or unstimulated CD4 T cells. Stimulation with PMA/ionomycin was used as a positive control, whereas thymidine incorporation. DC electroporated with MCF-7 RNA, A498 tumor cells process the same set of viral proteins in quantitatively different ways, resulting in the expansion of selective CTL specificities (40). Therefore, whole tumor RNA transduced DC might present a set of epitopes that partially differs from that presented by tumor cells of different origin.

Acknowledgments
We thank Sylvia Stephan and Bruni Schuster for excellent technical assistance.

References
16. Tjandrawan, A., A. W. Goldrath, E. A. Butz, S. Martin, and M. J. Bevan. 1997. Adenovirus-mediated delivery of antigenic epitopes into DC by a means of CTL induction. J. Immunol. 158:3270. FIGURE 3. Induction of Th cell responses by DC transduced with MCF-7 tumor RNA. CD4+ T lymphocytes were isolated using magnetic bead technology. Th cell induction was performed by coincubating 5 x 10^6 DC electroporated with MCF-7 total RNA and 2.5 x 10^6 autologous CD4+ T lymphocytes. Two restimulations with 2.5 x 10^6 autologous DC were performed on days 7 and 14 after T cell induction. IL-2 was added every second day after the first restimulation. The Ag specificity of the induced CD4+ T cells was measured on day 20 after T cell induction using a [3H]thymidine proliferation assay. CD4+ T cells (2 x 10^4) were coincubated with 10^5 DC for 5 days. The induced proliferation was determined on day 5 by a 16-h pulse with [3H]thymidine and subsequent measurement of thymidine incorporation. DC electroporated with MCF-7 RNA, A498 RNA, or irrelevant EGFP RNA were used as stimulators in the assay. Stimulation with PMA/ionomycin was used as a positive control, whereas DC electroporated with EGFP RNA or unstimulated CD4+ T cells were included as negative controls. HLA class II blocking was achieved by incubating DC with the mAb Tu39 (directed against HLA class II molecules) for 1 h before stimulation of CD4+ T cells.


