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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 Journal of Immunology, 2003, 170: 5892–5896.

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4 Abbreviations used in this paper: DC, dendritic cell; EGF, enhanced green fluorescent protein; RCC, renal cell carcinoma; TAA, tumor-associated Ag.

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.
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/Paque (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 × 10^7 cells/3 ml/well) into six-well plates (Corning, Cambridge, MA) in RPMI 10 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 10 medium supplemented with the following cytokines: human recombinant human rIL-2 (Genzyme, Cambridge, MA) for the generation of immature DC. For maturation, DC were additionally cultured with TNF-α (10 ng/ml; Genzyme) 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-Immuno-teck, 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 RNase 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 assessed 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 RP10 medium and returned to the incubator. The viability of the cells was above 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 RP10 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 RP10 medium. Additional DC transfected with RNA were treated as described below. After transfection, DC were incubated for 24 h in RP10 medium containing 10 ng/ml TNF-α for maturation of DC. For Th cell induction, 2.5 × 10^5 CD4^+ T cells were transfected with MCF-7 tumor RNA by electroporation on day 6 as described above. After transfection, DC were incubated for 24 h in RP10 medium containing 10 ng/ml TNF-α for maturation of DC. For Th cell induction, 2.5 × 10^5 CD4^+ T lymphocytes were co-cultured with 5 × 10^6 autologous DC (transfected with MCF-7 tumor RNA). On days 7 and 14 after T cell induction, restimulations were performed using 5 × 10^6 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 assayed on day 20 after T cell induction in a ^[3]H]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 10^5 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 molecules was achieved by incubating DC for 1 h before the assay with either either mAb W6/32 (20 μg/ml) directed against HLA class I molecules or mAb Tu39 (20 μg/ml) directed against HLA class II molecules (both Abs were provided by S. Stevanovic, University of Tübingen). Thymidine incorporation was measured on day 5 by a 16-h pulse with ^[3]H]thymidine (1 μCi/well; Amersham International, Little Chalfont, U.K.).

Results and Discussion

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^+ 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 specifically recognized MCF-7 cells and autologous DC electroporated with MCF-7-RNA, but not control cell lines or untreated DC in a standard ^[51]Cr 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 ^[51]Cr, 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 used target cells pulsed with the antigenic MUC1 (M1.1 and M1.2) or Her-2/neu (E75 and GP-2)-derived 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
With 10^5 DC for 5 days. The induced proliferation was determined on DC electroporated with EGFP RNA or unstimulated CD4^+ cells. Th cell induction was performed by coincubating 5 x 10^5 DC electroporated with MCF-7 RNA, A498 tumor RNA, CD4^+ T cells, which were entirely HLA class II restricted, exhibited a cross-reactivity to the Ags derived from the A498 RCC line, and induces Ag-specific CTL. The demonstration that electroporation of DC with whole tumor RNA results 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 the tumor and induces Ag-specific CTL recognizing only a fraction of the epitopes expressed on tumor cells.

Another interesting and important finding of our study is the demonstration that electroporation of DC with whole tumor RNA can elicit HLA class II-mediated T cell responses. Similar to the results obtained with HLA class I-restricted CTL, the induced CD4^+ T cells, which were entirely HLA class II restricted, exhibited a cross-reactivity to the Ags derived from the A498 RCC line, indicating that HLA class II epitopes might be shared among tumor cells of different origin.

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