Human Fibroblasts Transduced with CD80 or CD86 Efficiently *trans*-Costimulate CD4$^+$ and CD8$^+$ T Lymphocytes in HLA-Restricted Reactions: Implications for Immune Augmentation Cancer Therapy and Autoimmunity[1]

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Augmenting immunogenicity by genetically modifying tumor cells to express costimulatory molecules has proven to be a promising therapeutic strategy in murine tumor models and is currently under investigation in human clinical trials for metastatic cancer. However, there are significant technical and logistic problems associated with implementing strategies requiring direct gene modification of primary tumor cells. In an effort to circumvent these problems, we are developing a strategy in which the costimulatory signal required for tumor-specific T lymphocyte activation is provided by a genetically modified human fibroblast (*trans*-costimulation). We have evaluated the efficiency of CD80- and CD86-mediated *trans*-costimulation in the activation of human CD8$^+$ and CD4$^+$ T lymphocytes in MHC class I- and class II-restricted lymphoproliferation reactions. Our studies demonstrate that the efficiency of CD80- or CD86-mediated *trans*-costimulation of purified human CD8$^+$ and CD4$^+$ T lymphocytes is comparable to *cis*-costimulation under defined conditions. Moreover, a dose-response relationship consistent with the predicted two-hit kinetics of the reaction was evident in *trans*-costimulation reactions in which the ratio of target cells expressing either signal 1 or signal 2 was varied incrementally from 1:10 to 10:1. Importantly, the level of cell-surface CD86 required for *trans*-costimulation is equivalent to that constitutively expressed by human peripheral blood monocytes. These results may have significant implications for the clinical implementation of this type of cancer immunotherapy and also raise questions about the possibility of *trans*-costimulating autoreactive T lymphocytes in vivo. ***The Journal of Immunology*, 1999, 163: 3239–3249.**

Recent advances in our understanding of the process of T lymphocyte activation have led to the development of novel strategies for manipulating the immune system to augment antitumor effector functions. One of the most promising strategies is based on genetically modifying tumor cells to express the costimulatory molecules, CD80 (B7.1) and CD86 (B7.2), to enhance their immunogenicity. CD80 and CD86 are type I integral membrane glycoproteins that are present on the surface of activated T lymphocytes, B lymphocytes, and APC (1–9). In the process of T lymphocyte activation, which requires both an Ag-dependent and Ag-independent signal be delivered to the T lymphocyte, CD80 and CD86 provide the Ag-independent, or costimulatory, signal. The costimulatory signal is delivered when CD80 and CD86 interact as low-affinity ligands, or counter-receptors for CD28, or as high-affinity counter-receptors for CTLA-4 on the T lymphocyte surface (1–10). Functionally, CD86 is thought to be more important in the initiation phase of a cell-mediated immune response, and CD80 is thought to be more important in the maintenance and/or modulation of the immune response (1, 2, 5–9, 11, 12).

A number of reports have been published that describe the strategy of genetically manipulating cancer cells to express CD80 or CD86 in an effort to enhance tumor immunogenicity (4, 13–25). The hypothesis underpinning these studies is that gene-modified tumor cells expressing CD80 or CD86 can simultaneously present both Ag-specific and costimulatory activation signals to T lymphocytes, thereby obviating the need for an APC-mediated interaction. To date, the results of these studies have been promising in terms of inducing therapeutic and protective immune responses. It has been demonstrated that mice immunized with tumors expressing CD80 or CD86 develop a cell-mediated immune response that not only rejects unmodified tumor cells on rechallenge, but also induces long-term immunological memory (4, 13, 18). Moreover, under defined conditions this cellular vaccine strategy can also reduce the incidence and severity of metastatic disease (4). Collectively, these studies support the contention that rendering tumor cells immunogenic and recognizable to the immune system by manipulating costimulation of T lymphocyte activation is a viable and potentially therapeutic means of eliciting an effective CTL response for the treatment of minimal-residual and metastatic disease. We are interested in developing this type of cancer immunotherapy for the treatment of pediatric malignancies.

The technical difficulties associated with direct gene modification of tumor cells that we and others have encountered in attempting to translate the murine studies into clinically relevant human experimentation are significant. At each step in the process, which includes identifying suitable tumor types for ex vivo manipulation, establishing and expanding the tumor cell populations in short- and

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Received for publication February 19, 1999. Accepted for publication July 6, 1999.

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[1] This work was supported in part by a grant from the New Children's Hospital Annual Grants Scheme (CHF9716).

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 0022-1767/99/$02.00

long-term in vitro cultures, genetically modifying the tumor cells, and finally selecting the gene-modified population for further testing, there are potential problems. To circumvent these obstacles, we have focussed on developing an alternative strategy for providing the Ag-specific and costimulatory signals necessary for effector T lymphocyte activation. The strategy we are pursuing is based on the concept of providing the Ag-specific and costimulatory signals necessary for T lymphocyte activation from separate cells (i.e., in *trans*), in contrast to the strategy of modifying tumor cells to express both signals (i.e., in *cis*). Specifically, the tumor cell would continue to provide the Ag-specific signal to the T lymphocyte, while a nonneoplastic bystander cell, such as a fibroblast transduced to express CD86, would provide the costimulatory signal.

Although recent studies have attempted to show *trans*-costimulation of human T lymphocytes in vitro, the efficiency of the reaction remains controversial. In all but one study (26), the focus has been on activating $CD4^+$ T lymphocytes using CD80 as the costimulatory molecule (26–32). In the study by Cardoso et al. (26) in which $CD8^+$ T lymphocytes were examined, the investigators reported that *trans*-costimulation was ~10- to 30-fold less efficient than *cis*-costimulation for T lymphocyte activation and proliferation (26). Consistent with these findings, Liu and Janeway reported that CD80-mediated *trans*-costimulation of $CD4^+$ T lymphocyte activation was at least 80-fold less efficient than *cis*-costimulation and did not result in proliferation of the T lymphocyte population (28). However, in contrast, Ding and Shevach reported that *trans*-costimulation and *cis*-costimulation were equally efficient in the process of $CD4^+$ T lymphocyte activation (31). They also reported that *trans*-costimulation could activate both memory $CD4^+$ and naive $CD4^+$ T lymphocytes. This latter observation was inconsistent with the findings of Van de Velde and coworkers who reported that *trans*-costimulation was only effective for memory $CD4^+$ T lymphocyte activation and did not work at all with naive $CD4^+$ T lymphocytes (30).

Further complicating the question of *trans*-costimulation efficiency was the routine use of xenogeneic cells as a source of the *trans*-costimulatory signal in the assay systems described above. In summary, these in vitro studies have described *trans*-costimulation of murine $CD4^+$ T lymphocytes by monkey COS cells expressing human CD80 (28) and *trans*-costimulation of human $CD4^+$ T lymphocytes by murine 3T6 fibroblasts expressing CD80 (30), murine DAP.3 fibroblasts expressing CD80 (32), or Chinese hamster ovary (CHO)[3] cells expressing CD80 and/or CD86 (26, 29). These reports did not address the potential immunogenicity of the genetically modified xenogeneic cells or the fact that the xenogeneic cells do not express species-specific cell-surface receptors, ICAMS, and other minor costimulatory molecules that may be crucial for complete T lymphocyte activation.

To avoid the complicating issues inherent in the use of xenogeneic cells to provide *trans*-costimulatory signals, we have used allogeneic and autologous human fibroblasts to directly compare CD80- and CD86-mediated *cis*-costimulation with *trans*-costimulation of purified human T lymphocytes in a standardized lymphoproliferation assay. Moreover, in contrast to the previous reports we have studied *cis*-costimulation and *trans*-costimulation of both $CD4^+$ and $CD8^+$ T lymphocytes, because an understanding of the responses of both cell types will be of utmost importance in the development of this type of cancer immunotherapy strategy. Our studies reveal that T lymphocyte activation occurs with comparable efficiency when the Ag-specific signal and the CD80- or CD86-mediated costimulatory signals are provided by the same cell (*cis*-costimulation) or individually by two independent cells (*trans*-costimulation) under the assay conditions described. A dose-response relationship was observed when the ratio of cells expressing the Ag-specific signal (MHC class I or class II) to cells expressing the *trans*-costimulatory signal (CD80 or CD86) was varied incrementally from 1:10 to 10:1 for both $CD8^+$ T lymphocytes in a MHC class I-restricted lymphoproliferation assay and $CD4^+$ T lymphocytes in a MHC class II-restricted reaction. Furthermore, data from these titration experiments conformed to the "one-hit" and "two-hit" kinetics inherent in the *cis*-costimulation and *trans*-costimulation reactions, respectively. Collectively, these results may have significant implications for the increasing number of clinical trials aimed at evaluating this type of cancer immunotherapy and also raise a series of questions about the mechanisms that may regulate *trans*-costimulation in vivo to minimize autoimmune disease.

Materials and Methods

Fibroblast cell culture

Primary human fibroblasts were collected with informed consent from a healthy female donor by a routine full-thickness skin biopsy of the shoulder. The biopsy tissue was immediately placed into 500 μl of DMEM (Life Technologies, Grand Island, NY) supplemented with 20% (v/v) heat-inactivated bovine calf serum (Starrate Pty., Bethungra, Australia), 50 IU/ml penicillin, 50 μg/ml streptomycin (MultiCel, Trace Biosciences, Castle Hill, NSW, Australia), 50 μg/ml gentamicin (Sigma Aldrich, Irvine, U.K.), and 0.1 μg/ml Fungizone (Life Technologies). The tissue was minced finely, and the cell suspension was spread gently over the tissue culture surface of two 25-cm^2 flasks (Becton Dickinson, Franklin Lakes, NJ) and incubated at 37°C for 10 min in a 5% CO_2-humidified incubator to promote adherence. An additional 4 ml of a 1:1 mixture of Chang's complete medium and DMEM, supplemented with 20% (v/v) heat-inactivated bovine calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, and 0.1 μg/ml Fungizone was then added. The cultures were incubated for 5 days, after which an additional 2 ml of fresh medium was added, and the cultures were continued for another 7 days. Nonadherent cells were then removed, and the established donor skin fibroblasts (designated DS-fibroblasts) were passaged in culture as necessary using trypsin/EDTA (1:250) (MultiCel, Trace Biosciences) and maintained in DMEM supplemented with 10% (v/v) heat-inactivated bovine calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, and 0.1 μg/ml Fungizone. Primary human embryonic lung fibroblast cells (MRC-5, CCL-171, American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% (v/v) heat-inactivated bovine calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO_2-humidified incubator and passaged as necessary using trypsin/EDTA.

Primary human tumor cell isolation and in vitro culture

Tumor tissue samples were obtained, with appropriate Institutional Ethics Committee approval, from excess biopsy material collected from patients for routine diagnostic pathology. The tumor tissue samples were washed in betadine antiseptic solution (Faulding Pharmaceuticals, Salisbury, SA, Australia), rinsed thoroughly in HBSS (MultiCel, Trace Biosciences) containing 50 IU/ml penicillin and 50 μg/ml streptomycin (HBSS-P/S). The tumor tissue was transferred to a sterile petri dish and minced until fragments were ~1 mm^3 in size. The tissue was then either forced through a Falcon cell strainer (70-μm pore size nylon mesh, Becton Dickinson) or dissociated by treatment with 1 mg/ml collagenase/dispase (Boehringer Mannheim Australia, New South Wales, Australia) in HBSS-P/S in a 37°C shaker for 1 h. Cells were harvested from the supernatant, and any remaining tissue was treated with collagenase/dispase solution containing 0.1 μg/ml DNase 1 (Boehringer Mannheim, Indianapolis, IN), for a further 30–60 min. Cell suspensions, and any remaining tissue clumps, were then

[3] Abbreviations used in this paper: CHO, Chinese hamster ovary; HBSS-P/S, HBSS containing penicillin and streptomycin; DS-fibroblasts, donor skin fibroblasts; MFI, mean fluorescence intensity; ASPS, aveolar soft part sarcoma; ASPS/X, LXSN-transduced ASPS; ASPS/86, L86SN-transduced ASPS; MRC/X, LXSN-transduced MRC-5 fibroblasts; MRC/II/X, LXSN-transduced MRC-5 fibroblasts treated with IFN-γ to induce MHC class II; MRC/86, L86SN-transduced MRC-5 fibroblasts; MRC/II/86, L86SN-transduced MRC-5 fibroblasts treated with IFN-γ to induce MHC class II; DS/X, LXSN-transduced DS-fibroblasts; DS/86, L86SN-transduced DS-fibroblasts; MRC/80, 80SN-transduced MRC-5 fibroblasts.

plated into Lab-Tek glass chamber slides (Nalge Nunc International, Naperville, IL) or Falcon Multiwell six-well flat-bottom tissue culture plates (Becton Dickinson) coated with either Matrigel basement membrane matrix (Becton Dickinson) diluted 1:8 in DMEM or 4.5 μg/cm^2 poly-D-lysine hydrobromide, high m.w. (Becton Dickinson). Alternatively, cells were plated into uncoated Falcon Primaria multiwell six-well flat-bottom tissue culture plates (Becton Dickinson). Cultures were left undisturbed for 3–5 days before nonadherent cells were removed, and the established culture was passaged as necessary using trypsin/EDTA and maintained in DMEM supplemented with 20% (v/v) heat-inactivated bovine calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, and 0.1 μg/ml Fungizone.

Retrovirus vector constructs and transduction of cells

Human CD80 cDNA (Ref. 1; GenBank accession no. M27533) and CD86 cDNA (Ref. 6, GenBank accession no. L25259) were kindly provided by Dr. Gordon Freeman (Dana-Farber Cancer Institute, Harvard Medical School, Boston. MA) in pCDM8. CD80 and CD86 cDNA sequences were subcloned into the retrovirus vector plasmid pLXSN (GenBank accession no. M28248) (33) to generate pL80SN and pL86SN as described previously (34). The production of ecotropic and amphotropic retrovirus from the pL80SN and pL86SN plasmids and the protocol used for transduction of cells was performed as described (34) with the following modifications. Supernatant from L80SN, L86SN, or LXSN amphotropic retrovirus producer cell lines was used to transduce MRC-5 fibroblasts, DS-fibroblasts, and primary tumor samples. The producer cell line supernatants were harvested, mixed with 4 μg/ml protamine sulfate (Fisons Pharmaceuticals, Sydney, Australia) and filtered through a Minisart 0.45-μm pore size filter (Sartorius, Gottingen, Germany) to remove cell debris. Culture medium was aspirated from the target cells, and the retrovirus-containing supernatant was added immediately. The volume of retrovirus supernatant added to the cultures varied from 2 ml per well of a 6-well plate to 5 ml per 25-cm^2 flask and 10 ml per 75-cm^2 flask. The titer of each supernantant was $\sim 1 \times 10^6$ CFU/ml when titered on NIH-3T3 cells as described (35). In general, this procedure was repeated four times over 48 h. Cells were then washed, and cultures were continued in the appropriate growth medium for up to 7 days before the media was replaced and supplemented with G-418 (Boehringer Mannheim) at a concentration of 350 μg/ml (active drug) for primary tumor cell cultures (the primary tumor cells were only transduced with LXSN or L86SN for the subsequent studies, not with L80SN) or 700 μg/ml (active drug) for fibroblast cultures for a further 14 days to select transduced cells. Flow cytometry and immunofluorescence confocal microscopy was performed to verify CD80 or CD86 expression before and after G-418 selection.

Flow cytometry and immunofluorescence confocal microscopy

Flow cytometry was performed on a FACScan cytometer (Becton Dickinson, Mountain View, CA), according to the manufacturer's instructions, and immunofluorescence confocal microscopy was performed using a Leica CLSM confocal microscope (Leica, Deerfield, IL) as described (34). Mean fluorescence intensity (MFI) was calculated for each FACScan profile. Murine Abs used for the study were anti-CD80 and anti-CD86 (PharMingen, San Diego, CA), anti-CD4-FITC and anti-CD8-PE conjugates (Becton Dickinson), anti-HLA-A, B, C, anti-HLA-DP, DQ, DR, anti-CD14-PE, and anti-CD19-PE (Dako Botany, NSW, Australia). Unconjugated Abs were visualized by incubation with FITC-conjugated goat anti-mouse Ig (Becton Dickinson).

Isolation of CD4$^+$ and CD8$^+$ T lymphocytes

Freshly isolated PBMC were separated by Ficoll-Hypaque gradient sedimentation of heparin-treated blood samples from healthy donors as described (34). CD4$^+$ T lymphocytes were then isolated from the PBMC fraction using M-450 CD4 (T helper/inducer) Dynabeads and DETACHaBEAD (Dynal, Oslo, Norway), according to the manufacturer's protocols. Similarly, CD8$^+$ T lymphocytes were isolated from the PBMC fraction using M-450 CD8 Dynabeads and DETACHaBEAD. The purity of the isolated CD4$^+$ and CD8$^+$ T lymphocyte populations was monitored by flow cytometry.

Induction of MHC class II expression on fibroblasts by IFN-γ and TNF-α treatment in vitro

As necessary, LXSN-transduced, L80SN-transduced, L86SN-transduced, and untransduced MRC-5 and DS-fibroblasts were incubated with 10 ng/ml IFN-γ (Genzyme, Cambridge, MA), 10 ng/ml TNF-α (Genzyme), or both cytokines for 72 h in DMEM supplemented with 10% heat-inactivated bovine calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cells were then washed in situ with PBS and incubated for a further 24 h in fresh medium at 37°C in a 5% CO_2-humidified incubator. Samples of the cytokine-treated fibroblasts were then examined by flow cytometry for MHC class I (HLA-A, B, C), MHC class II (HLA-DP, DQ, DR), CD80, and CD86 surface expression before inclusion in the lymphocyte proliferation assays.

Lymphocyte proliferation assay using allogeneic, monocyte-depleted PBMC

Lymphocyte proliferation assays were performed in 96-well tissue culture plates as described above with the following modifications. PBMC were separated by Ficoll-Hypaque gradient sedimentation of heparin-treated blood samples from healthy donors, washed several times with PBS, and the CD14$^+$ monocytes/macrophages were depleted by adherence panning for 4 h in a T-75 flask at 37°C in a 5% CO_2-humidified incubator. A sample of the nonadherent APC-depleted PBMC was then examined by FACScan to determine the mononuclear cell profile and the degree of depletion of CD14$^+$ cells. The nonadherent, monocyte-depleted PBMC (2×10^5 PBMC/well) were then incubated with γ-irradiated untransduced, LXSN-transduced, or L86SN-transduced primary tumor cells at a fixed E:T ratio of 20:1. After 96 h incubation at 37°C in a 5% CO_2-humidified incubator, cultures were pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine for 18 h, then harvested onto glass-fiber filters, where [^{3}H]thymidine incorporation was determined by liquid scintillation counting. Assays were performed in triplicate.

T lymphocyte proliferation assays using purified CD4$^+$ and CD8$^+$ T lymphocytes at defined E:T cell ratios

T lymphocyte proliferation assays were performed in 96-well tissue culture plates in DMEM supplemented with 10% heat-inactivated human AB serum (Sigma, St. Louis, MO), 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO_2-humidified incubator. The E:T ratio for each assay was maintained at 10:1 in 200 μl final volume, and in the dose-response studies the ratio of target cells expressing either signal 1 or signal 2 was incrementally varied from 1:10 to 10:1, as indicated. The purified T lymphocytes were incubated with various target cells alone or in combination with untreated, LXSN-transduced, L80SN-transduced, L86SN-transduced, and/or IFN-γ-treated primary tumor cells, DS-fibroblasts, or MRC-5 fibroblasts as indicated. The target cells were gamma-irradiated with 500 rad and then dispensed in 50 μl volume at a final concentration of 2×10^4 cells per well and allowed to adhere for 4 h. The purified CD4$^+$ or CD8$^+$ T lymphocyte effector cells were then added in 50 μl volume at a concentration of 2×10^5 cells per well. After 96 h incubation at 37°C in a 5% CO_2-humidified incubator, the cultures were pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine (NEN Life Science Products, Boston, MA) for 18 h, and the cells then harvested onto glass-fiber filters using a Packard Filtermate 196 harvester (Packard, Meriden, CT). The [^{3}H]thymidine incorporation was determined by liquid scintillation counting in a Tri-Carb 4000 series counter (Packard). All assays were performed in triplicate.

Results

Transduction of primary tumor cell cultures and fibroblasts with LXSN, L80SN or L86SN

Of the 41 primary tumor tissue samples that were obtained, in vitro culture of viable cells was established with 27 of the samples, as defined by the tumor cells completing at least one round of replication (i.e., doubling) in vitro. Of these 27 tumor cultures, only 12 continued to replicate beyond the first doubling, a phenotype required for retrovirus transduction to be attempted. These 12 tumor samples were incubated with amphotropic L86SN, and then examined by immunofluorescence confocal microscopy for CD86 surface expression (Table I). Seven of the 12 tumors were transduced to express CD86 with an efficiency that was consistently $\leq$5% (i.e., 5% or fewer cells expressed CD86 by immunofluorescence confocal microscopic analysis), and an example of an L86SN-transduced mesoblastic nephroma is shown (Fig. 1*A*). One of the 12 tumor samples, an alveolar soft part sarcoma (ASPS) was initially transduced by L86SN with an efficiency approaching 70% (Fig. 1*B*), indicating that the viral titer was not the limiting factor for transduction efficiency. The ASPS tumor and a mesoblastic nephroma sample were the only L86SN-transduced tumor samples

Table I. *In vitro growth, L86SN-transduction, and G-418 selection of primary human tumor cells*

Tumor Type	Total Samples	No. Cultures Established	No. L86SN Treated[a]	No. Successful Transductions[b]	G-418 Selection[c]
ASPS	1	1	1	1	1/1
Ewings	4	3	2	1	–
Neuroblastoma	9	7	2	2	–
Wilms	6	5	2	0	–
Mesoblastic nephroma	2	2	2	2	0/1
Retinoblastoma	1	1	1	0	–
Astrocytoma	3	3	1	1	–
Hodgkin's lymphoma	1	1	1	1	–
Others	14	4	–	–	–
Total	41	27	12	8	1/2

[a] Only tumor samples that continued to replicate in vitro after the initial culture was established were incubated with L86SN retrovirus for transduction.
[b] The success of L86SN transduction was assessed by immunofluorescence confocal microscopy with anti-CD86 Ab. Only tumor samples that were positive for CD86, and continued to replicate in vitro after transduction, were then subjected to G-418 selection.
[c] Number of tumor samples viable after G-418 selection/number of tumor samples subjected to G-418 selection.

that continued to divide in culture after transduction, and therefore both samples were subjected to G-418 selection. Only the L86SN-transduced ASPS survived G-418 selection and were subjected to further analysis in the PBMC lymphoproliferation assay.

In a parallel series of experiments, the DS-fibroblasts and MRC-5 fibroblasts, which replicated well in vitro, were also transduced with L80SN, L86SN, or LXSN. The initial transduction efficiency for both fibroblast populations reproducibly ranged from 5 to 20% (Fig. 1*C*). In contrast with the primary tumor samples, the L80SN-, L86SN-, and LXSN-transduced DS-fibroblasts and MRC5 fibroblasts continued to grow in vitro after transduction and were readily selected with G-418 for use in subsequent lymphoproliferation assays.

PBMC proliferation in response to CD86-mediated costimulation by L86SN-transduced primary tumor cells

To determine whether L86SN-transduced primary ASPS cells expressing CD86 could activate allogeneic T lymphocytes by providing both the Ag-specific and costimulatory signals on the same cell (i.e., in a *cis* configuration), 2×10^5 monocyte-depleted PBMC were incubated with untransduced ASPS (designated as ASPS), LXSN-transduced ASPS (designated as ASPS/X), or L86SN-transduced ASPS (designated as ASPS/86) target cells in triplicate at an E:T ratio of 20:1. A typical experimental result is shown in Fig. 2. Comparison of the [^{3}H]thymidine incorporation by monocyte-depleted PBMC exposed to ASPS, ASPS/X, or ASPS/86 revealed that CD86 expression resulted in significant allogeneic PBMC proliferation. This experiment was performed in triplicate a total of six times with monocyte-depleted PBMC from two individual allogeneic donors, and the results were reproducible. The experiment could not be performed with autologous PBMC because the patient was undergoing chemotherapy.

MHC class I, class II, and CD86 expression on fibroblast target cells

Defined target cell populations required for the various T lymphocyte proliferation assays were characterized by FACScan analysis before inclusion in the assays. For the $CD8^+$ T lymphocyte proliferation assays, MHC class I expression was required, and FACScan analysis demonstrated that both MRC-5 and DS-fibroblasts stably express endogenous MHC class I (HLA-A, B, C) (Fig. 3, *A* and *B*, respectively). To evaluate costimulation of $CD4^+$ T lymphocytes, we required an allogeneic target cell population expressing MHC class II (HLA-DP, DQ, DR). Although it is well established that treating human fibroblasts with IFN-γ induces MHC class II expression, a recent report indicated that treating murine fibroblast-like cells with a combination of IFN-γ and TNF-α would also induce CD80 (but not CD86) expression (36). We tested this by treating MRC-5 fibroblasts with IFN-γ and TNF-α as described (36). In contrast to the previous report, neither CD80 or CD86 expression was induced after pretreatment with either cytokine alone or in combination (not shown). However, MHC class II

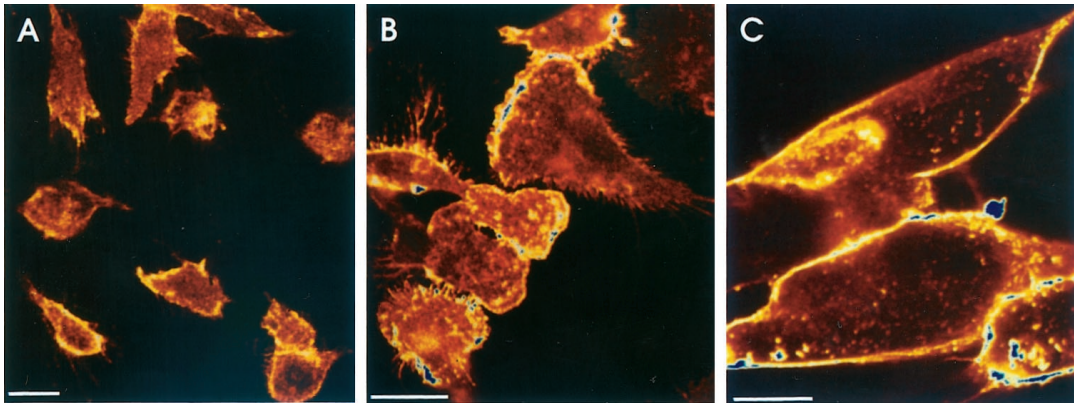

FIGURE 1. Retroviral transduction of primary human tumor cells and fibroblasts with L86SN results in stable surface membrane expression of CD86. Typical immunofluorescence confocal microscope images of (*A*) mesoblastic nephroma cells, (*B*) ASPS cells, and (*C*) primary human donor skin fibroblasts (DS-fibroblasts) after L86SN transduction and immunolabeling with anti-CD86 Ab and FITC-labeled goat anti-mouse Ig. Scale bar represents 20 μm.

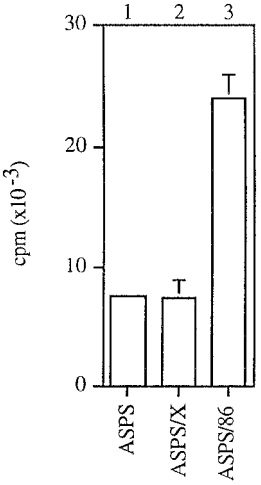

FIGURE 2. ASPS tumor cells transduced to express CD86 have increased immunogenicity in an in vitro allogeneic lymphocyte proliferation assay. [^{3}H]Thymidine uptake measuring proliferation of allogeneic, monocyte-depleted PBMC in response to incubation with untransduced ASPS cells (*column 1*), ASPS/X cells (*column 2*), or ASPS/86 cells (*column 3*). The experiment was performed in triplicate, and the mean cpm ± SEM is shown. The result is representative of six experiments with monocyte-depleted PBMC from two different allogeneic donors.

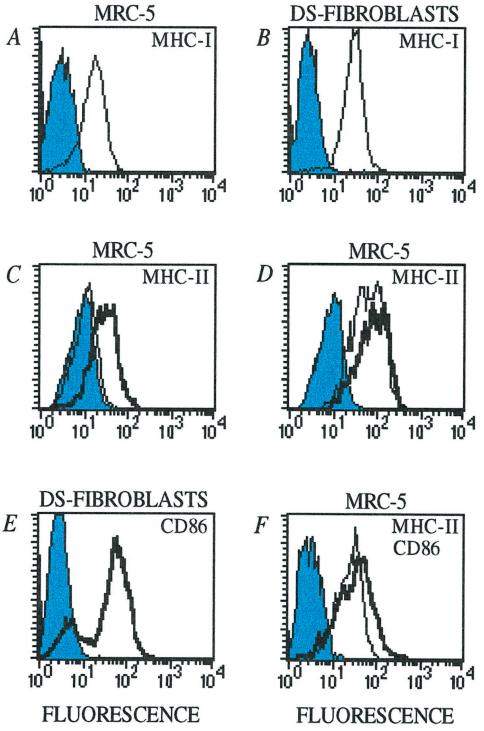

FIGURE 3. FACScan analysis of untreated, cytokine treated, and retrovirally transduced target cell populations used in the *cis*-costimulation and *trans*-costimulation of purified T lymphocyte populations. FACScan profiles of (*A*) MRC-5 fibroblasts labeled with HLA-A-, B-, and C-specific Ab, (*B*) DS-fibroblasts labeled with HLA-A-, B-, and C-specific Ab, (*C*) MRC-5 fibroblasts labeled with HLA-DP-, DQ-, and DR-specific Ab 24 h (thin line) or 48 h (thick line) after IFN-γ treatment, and (*D*) MRC-5 fibroblasts labeled with HLA-DP-, DQ-, and DR-specific Ab 72 h (thin line) or 96 h (thick line) after IFN-γ treatment, (*E*) L86SN-transduced DS-fibroblasts labeled with anti-CD86 Ab, and (*F*) L86SN-transduced and IFN-γ-treated MRC-5 fibroblasts labeled with anti-CD86 Ab (thick line) or HLA-DP-, DQ-, and DR-specific Ab (thin line). Isotype control Ab labeling is shaded.

expression was induced on MRC-5 fibroblasts (the allogeneic target cell population) by treatment with IFN-γ. A time-course study of the induction and persistence of MHC class II expression on MRC-5 fibroblasts was performed by FACScan analysis. MHC class II expression (HLA-DP, DQ, DR) was undetectable 24 h after exposure to IFN-γ (MFI = 12, isotype control MFI = 11) and present at moderate levels on the MRC-5 fibroblasts by 48 h (MFI = 29) (Fig. 3*C*). The surface expression peaked by 72 h (MFI = 63) and remained relatively stable from 72 to 96 h (Fig. 3*D*). Re-exposure to IFN-γ did not increase the level of MHC class II on the MRC-5 cell surface (not shown). After L86SN transduction and G-418 selection, the DS-fibroblasts and MRC-5 fibroblasts expressed equivalent levels of surface CD86 by FACScan analysis (Fig. 3, *E* and *F*, respectively). When the L86SN-transduced MRC-5 fibroblasts were treated with IFN-γ, both CD86 and MHC class II were simultaneously expressed (Fig. 3*F*). Similar results were obtained with the L80SN-transduced and G-418 selected DS-fibroblasts and MRC-5 fibroblasts (not shown).

The effects of gamma irradiation on endogenous and transgene expression in target fibroblasts

Because all target fibroblast populations were to be subjected to irradiation before inclusion in T lymphocyte proliferation assays, it was important to examine whether gamma irradiation 1) induced endogenous CD80, CD86, or MHC class II expression or 2) had any effect on the levels of CD80 or CD86 expression in L80SN- and L86SN-transduced cells, respectively. Untreated, L80SN-transduced, L86SN-transduced, and IFN-γ-treated MRC-5 and DS-fibroblasts were subjected to 500 rad of gamma irradiation and then analyzed by FACScan. These studies revealed that gamma irradiation did not induce endogenous CD80, CD86, or MHC class II expression on any of the target cell populations and did not alter the level of CD80 or CD86 expression in L80SN- or L86SN-transduced cells, respectively, or had any effect on the MHC class II expression of IFN-γ-treated fibroblasts (not shown).

Analysis of CD80-mediated and CD86-mediated cis-*costimulation and* trans-*costimulation of CD4$^+$ T lymphocytes in an MHC class II-restricted reaction*

Activation of human CD4$^+$ T lymphocytes requires that they receive two signals, an Ag-specific signal presented in the context of an MHC class II complex, and a second costimulatory signal. In this study, CD80 or CD86 was used to provide the costimulatory signal either 1) on the same allogeneic target cell as the Ag-specific signal (i.e., *cis*-costimulation) or 2) in a *trans*-configuration from a separate, bystander fibroblast (i.e., *trans*-costimulation). The CD4$^+$ T lymphocytes used in the lymphoproliferation assay were purified from donor PBMC, and FACScan analysis revealed the population to be ≥96% CD4$^+$ T lymphocytes, with no CD8$^+$ T lymphocytes, CD14$^+$ monocyte/macrophage, or CD19$^+$ B lymphocytes detectable.

For the *cis*-costimulation reaction, shown in Fig. 4*A*, allogeneic CD4$^+$ T lymphocytes were incubated with 1) MRC-5 fibroblasts transduced with LXSN, designated MRC/X, 2) MRC-5 fibroblasts transduced with LXSN then treated with IFN-γ to induce MHC class II expression, designated MRC/II/X, 3) MRC-5 fibroblasts transduced with L86SN, designated MRC/86, or 4) MRC-5 fibroblasts transduced with L86SN then treated with IFN-γ to induce MHC class II expression, designated MRC/II/86. A comparison of the [^{3}H]thymidine incorporation in the *cis*-costimulation reaction

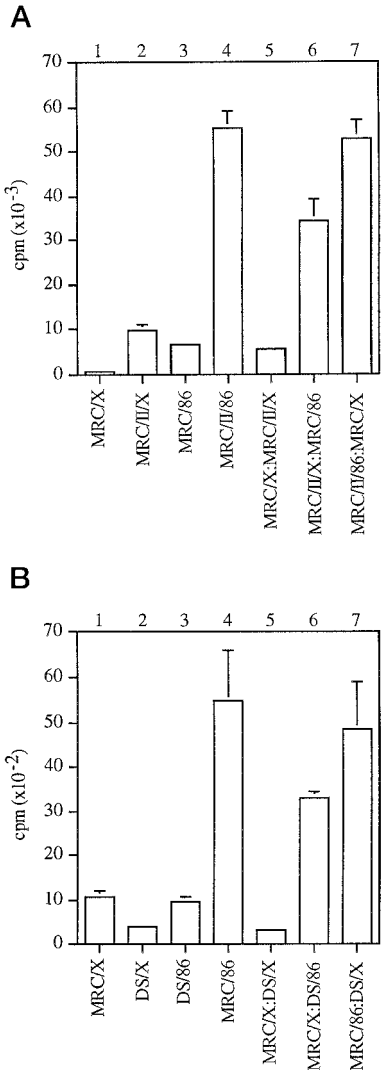

FIGURE 4. CD86-mediated *cis*-costimulation and *trans*-costimulation of (*A*) purified $CD4^+$ T lymphocytes and (*B*) $CD8^+$ T lymphocytes in MHC class II- and class I-restricted reactions, respectively. *A*, CD86-mediated costimulation was examined by incubating purified allogeneic $CD4^+$ T lymphocytes with MRC/X fibroblasts (*column 1*), MRC/II/X fibroblasts (*column 2*), MRC/86 fibroblasts (*column 3*), MRC/II/86 fibroblasts (*column 4*), or a 1:1 mixture of MRC/X and MRC/II/X fibroblasts (*column 5*), a 1:1 mixture of MRC/II/X and MRC/86 fibroblasts (*column 6*), or a 1:1 mixture of MRC/II/86 and MRC/X fibroblasts (*column 7*) in a [^{3}H]thymidine uptake assay. Each experiment was performed in triplicate, and the mean cpm ± SEM is shown. This result is representative of $n = 6$ experiments with $CD4^+$ T lymphocytes from three different allogeneic donors. *B*, CD86-mediated costimulation was examined by incubating purified $CD8^+$ T lymphocytes with allogeneic MRC/X fibroblasts (*column 1*), autologous DS/X fibroblasts (*column 2*), autologous DS/86 fibroblasts (*column 3*), allogeneic MRC/86 fibroblasts (*column 4*), or a 1:1 mixture of allogeneic MRC/X and autologous DS/X fibroblasts (*column 5*), a 1:1 mixture of allogeneic MRC/X and autologous DS/86 fibroblasts (*column 6*) or a 1:1 mixture of allogeneic MRC/86 and autologous DS/X fibroblasts (*column 7*) in a [^{3}H]thymidine uptake assay. Each experiment was performed in triplicate, and the mean cpm ± SEM is shown. This result is representative of three experiments.

with the control reactions revealed that the expression of CD86 in *cis*-configuration with MHC class II on MRC-5 fibroblasts resulted in significant proliferation of allogeneic $CD4^+$ T lymphocytes.

For the *trans*-costimulation studies, shown in Fig. 4*A*, allogeneic $CD4^+$ T lymphocytes were incubated with the following target cell populations: 1) a 1:1 mixture of MRC/X and MRC/II/X cells or 2) a 1:1 mixture of MRC/II/X and MRC/86. An additional *cis*-costimulation control for these reactions (1 and 2) was a 1:1 mixture of MRC/II/86 and MRC/X cells. A comparison of the [^{3}H]thymidine incorporation in the control reaction with the *trans*-costimulation reaction revealed that expression of CD86 on one half of the MRC-5 fibroblasts, and MHC class II on the remaining cells, resulted in significant *trans*-costimulation of allogeneic $CD4^+$ T lymphocytes. The level of proliferation of $CD4^+$ T lymphocytes in the *trans*-costimulation reaction was consistently within 60–65% of that observed in the *cis*-costimulation reactions. This experiment was repeated six times in triplicate with $CD4^+$ T lymphocytes from three different allogeneic donors, and the results were reproducible. For the *cis*-costimulation and *trans*-costimulation studies described above, the results shown are those obtained using L86SN-transduced target cells. However, the lymphoproliferation results are representative of those obtained with L80SN-transduced target cells in both magnitude and profile of response (not shown).

Analysis of CD80-mediated and CD86-mediated cis-*costimulation and* trans-*costimulation of $CD8^+$ T lymphocyte proliferation in an MHC class I-restricted reaction*

Having demonstrated that $CD4^+$ T lymphocyte proliferation can be induced by CD80-mediated and CD86-mediated *cis*-costimulation or *trans*-costimulation, the objective of this study was to determine the efficiency of activating $CD8^+$ T lymphocytes by 1) providing both the MHC class I-restricted Ag-specific signal and the CD80 or CD86 costimulatory signal on the same cell (*cis*-costimulation) or 2) providing the Ag-specific signal and the costimulatory signal on separate cells in a *trans* configuration (*trans*-costimulation). The $CD8^+$ T lymphocyte effector population were isolated from PBMC obtained from the donor who provided the DS-fibroblasts. FACScan analysis of the purified $CD8^+$ T lymphocytes revealed that the population was >96% $CD8^+$ T lymphocytes, and no $CD4^+$ T lymphocytes, $CD14^+$ monocyte/macrophage, or $CD19^+$ B lymphocytes were detectable.

For the *cis*-costimulation studies, shown in Fig. 4*B*, $CD8^+$ T lymphocytes were incubated with 1) allogeneic LXSN-transduced MRC-5 fibroblasts, designated MRC/X, 2) autologous LXSN-transduced DS-fibroblasts, designated DS/X, 3) autologous L86SN-transduced fibroblasts expressing CD86, designated DS/86, or 4) allogeneic L86SN-transduced MRC-5 fibroblasts expressing CD86, designated MRC/86. A comparison of the [^{3}H]thymidine incorporation in the *cis*-costimulation reaction with the control reactions revealed that the expression of CD86 in *cis*-configuration with MHC class I on the allogeneic MRC-5 fibroblasts resulted in significant proliferation of the $CD8^+$ T lymphocytes. This experiment was repeated three times in triplicate, and the results were reproducible.

For the *trans*-costimulation studies, shown in Fig. 4*B*, the donor $CD8^+$ T lymphocytes were incubated with the following target cell populations: 1) a 1:1 mixture of allogeneic MRC/X fibroblasts, and autologous DS/X fibroblasts, or 2) a 1:1 mixture of allogeneic MRC/X fibroblasts and autologous DS/86 fibroblasts. An additional *cis*-costimulation control for these reactions (1 and 2) was a 1:1 mixture of allogeneic MRC/86 fibroblasts and autologous DS/X fibroblasts. A comparison of the [^{3}H]thymidine incorporation in the control reaction with the *trans*-costimulation reaction revealed that expression of CD86 on autologous DS-fibroblasts, and MHC class I on allogeneic MRC/X fibroblasts resulted in significant *trans*-costimulation of autologous $CD8^+$ T lymphocyte effector cells. The level of proliferation of $CD8^+$ T lymphocytes in the *trans*-costimulation reaction was consistently within 60–70%

of that observed in the *cis*-costimulation reactions. This experiment was repeated three times in triplicate, and the results were reproducible. For the *cis*-costimulation and *trans*-costimulation studies described abovem the results shown are those obtained using L86SN-transduced target cells. However, the lymphoproliferation results are representative of those obtained with L80SN-transduced target cells in both magnitude and profile of response (not shown).

Analysis of the dose-response kinetics of CD80- and CD86-mediated trans*-costimulation of purified CD4$^+$ T lymphocyte proliferation in an MHC class II-restricted reaction*

As mentioned above, the *trans*-costimulation of T lymphocytes would be expected to demonstrate a two-hit kinetic reaction, in that the T lymphocyte would require contact with a cell providing signal 1 (antigenic signal) and a second cell providing signal 2 (costimulatory signal) to be activated. In the initial *trans*-costimulation reactions (described above), 50% of the target cells were expressing signal 1 (antigenic signal), and the remaining 50% were expressing signal 2 (costimulatory signal). To investigate the kinetics of the *trans*-costimulation of purified CD4$^+$ T lymphocytes, a series of dose-response reactions were prepared. In these reactions, the absolute number of target cells remained constant, but the ratio of target cells expressing signal 1 (antigenic signal) to those expressing signal 2 (costimulatory signal) was varied.

For CD80-mediated *trans*-costimulation of CD4$^+$ T lymphocytes in an MHC class II-restricted reaction, shown in Fig. 5*A*, 2×10^5 purified CD4$^+$ T lymphocytes were incubated with the following target cell populations: 1) 2×10^4 control MRC/II/X cells, 2) 2×10^4 L80SN-transduced MRC-5 Ribroblasts (MRC/80 cells), or a total of 2×10^4 target cells consisting of 3) a 1:10 mixture of MRC/II/X and MRC/80 cells, 4) a 1:2 mixture of MRC/II/X and MRC/80 cells, 5) a 1:1 mixture of MRC/II/X and MRC/80 cells, 6) a 2:1 mixture of MRC/II/X and MRC/80 cells, or 7) a 10:1 mixture of MRC/II/X and MRC/80 cells. A comparison of the [^{3}H]thymidine incorporation in the control reactions with the *trans*-costimulation reactions reveals a dose-response curve consistent with the two-hit kinetics of the reaction. This experiment was repeated three times in triplicate with CD4$^+$ T lymphocytes from two different donors, and the results were reproducible.

For the CD86-mediated *trans*-costimulation of CD4$^+$ T lymphocytes in an MHC class II-restricted reaction, shown in Fig. 5*B*, 2×10^5 purified CD4$^+$ T lymphocytes were incubated with the following target cell populations: 1) 2×10^4 control MRC/II/X cells, 2) 2×10^4 control MRC/86 cells, or a total of 2×10^4 target cells consisting of 3) a 1:10 mixture of MRC/II/X and MRC/86 cells, 4) a 1:2 mixture of MRC/II/X and MRC/86 cells, 5) a 1:1 mixture of MRC/II/X and MRC/86 cells, 6) a 2:1 mixture of MRC/II/X and MRC/86 cells, or 7) a 10:1 mixture of MRC/II/X and MRC/86 cells. A comparison of the [^{3}H]thymidine incorporation in the control reactions with the *trans*-costimulation reactions reveals a similar dose-response curve to that observed with CD80-mediated *trans*-costimulation of CD4$^+$ T lymphocytes. This experiment was repeated twice in triplicate with CD4$^+$ T lymphocytes from two different donors, and the results were reproducible.

Analysis of the dose-response kinetics of CD80- and CD86-mediated trans*-costimulation of purified CD8$^+$ T lymphocyte proliferation in an MHC class I-restricted reaction*

For the CD80-mediated *trans*-costimulation of CD8$^+$ T lymphocytes in an MHC class I-restricted reaction, shown in Fig. 6*A*, 2×10^5 purified CD8$^+$ T lymphocytes were incubated with the following target cell populations: 1) 2×10^4 control MRC/X cells, 2)

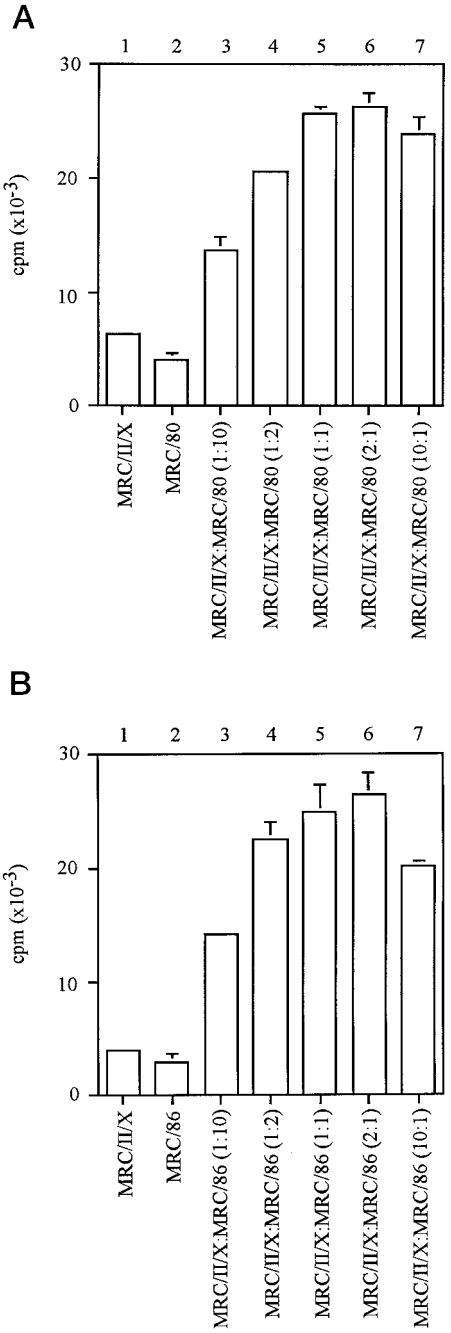

FIGURE 5. Dose-response kinetics of CD80- and CD86-mediated *trans*-costimulation of purified CD4$^+$ T lymphocyte proliferation in an MHC class II-restricted reaction. *A*, Purified CD4$^+$ T lymphocytes were incubated with MRC/II/X cells (*column 1*), MRC/80 cells (*column 2*), a 1:10 mixture of MRC/II/X and MRC/80 cells (*column 3*), a 1:2 mixture of MRC/II/X and MRC/80 cells (*column 4*), a 1:1 mixture of MRC/II/X and MRC/80 cells (*column 5*), a 2:1 mixture of MRC/II/X and MRC/80 cells (*column 6*), or a 10:1 mixture of MRC/II/X and MRC/80 cells (*column 7*) in a [^{3}H]thymidine incorporation assay. Each experiment was performed in triplicate, and the mean cpm ± SEM is shown. This result is representative of $n = 3$ experiments with CD4$^+$ T lymphocytes from two different donors. *B*, Purified CD4$^+$ T lymphocytes were incubated with MRC/II/X cells (*column 1*), MRC/86 cells (*column 2*), a 1:10 mixture of MRC/II/X and MRC/86 cells (*column 3*), a 1:2 mixture of MRC/II/X and MRC/86 cells (*column 4*), a 1:1 mixture of MRC/II/X and MRC/86 cells (*column 5*), a 2:1 mixture of MRC/II/X and MRC/86 cells (*column 6*), or a 10:1 mixture of MRC/II/X and MRC/86 cells (*column 7*) in a [^{3}H]thymidine incorporation assay. Each experiment was performed in triplicate, and the mean cpm ± SEM is shown. This result is representative of three experiments with CD4$^+$ T lymphocytes from two different donors.

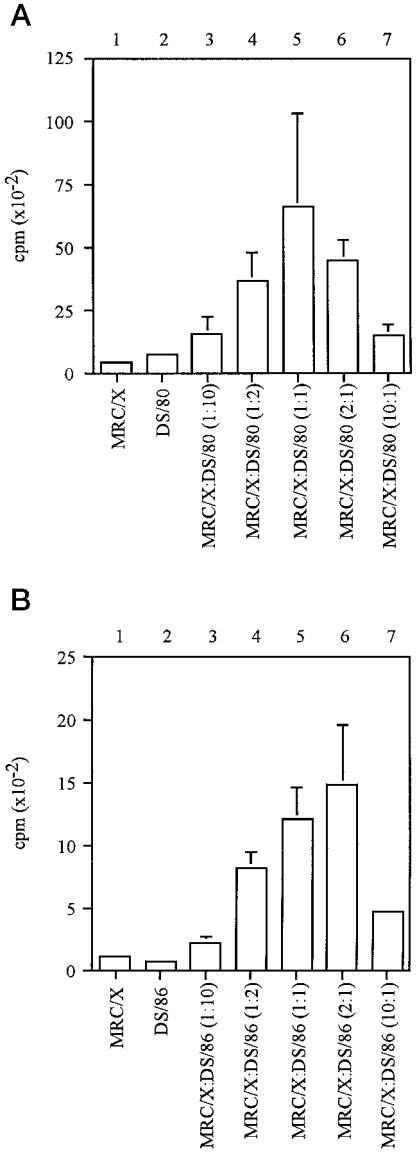

FIGURE 6. Dose-response kinetics of CD80- and CD86-mediated *trans*-costimulation of purified $CD8^+$ T lymphocyte proliferation in an MHC class I-restricted reaction. *A*, Purified $CD8^+$ T lymphocytes were incubated with MRC/X cells (*column 1*), DS/80 cells (*column 2*), a 1:10 mixture of MRC/X and DS/80 cells (*column 3*), a 1:2 mixture of MRC/X and DS/80 cells (*column 4*), a 1:1 mixture of MRC/X and DS/80 cells (*column 5*), a 2:1 mixture of MRC/X and DS/80 cells (*column 6*), or a 10:1 mixture of MRC/X and DS/80 cells (*column 7*) in a [^{3}H]thymidine incorporation assay. Each experiment was performed in triplicate, and the mean cpm ± SEM is shown. This result is representative of two experiments. *B*, Purified $CD8^+$ T lymphocytes were incubated with MRC/X cells (*column 1*), DS/86 cells (*column 2*), a 1:10 mixture of MRC/X and DS/86 cells (*column 3*), a 1:2 mixture of MRC/X and DS/86 cells (*column 4*), a 1:1 mixture of MRC/X and DS/86 cells (*column 5*), a 2:1 mixture of MRC/X and DS/86 cells (*column 6*), or a 10:1 mixture of MRC/X and DS/86 cells (*column 7*) in a [^{3}H]thymidine incorporation assay. Each experiment was performed in triplicate, and the mean cpm ± SEM is shown. This result is representative of two experiments.

2×10^4 control DS/80 cells, or a total of 2×10^4 target cells consisting of 3) a 1:10 mixture of MRC/X and DS/80 cells, 4) a 1:2 mixture of MRC/X and DS/80 cells, 5) a 1:1 mixture of MRC/X and DS/80 cells, 6) a 2:1 mixture of MRC/X and DS/80 cells, or 7) a 10:1 mixture of MRC/X and DS/80 cells. A comparison of the [^{3}H]thymidine incorporation in the control reactions with the *trans*-costimulation reactions reveals a dose-response curve consistent with the two-hit kinetics of the reaction. This experiment was repeated twice in triplicate, and the results were reproducible.

For the CD86-mediated *trans*-costimulation of $CD8^+$ T lymphocytes in an MHC class I-restricted reaction, shown in Fig. 6*B*, 2×10^5 purified $CD8^+$ T lymphocytes were incubated with the following target cell populations: 1) 2×10^4 control MRC/X cells, 2) 2×10^4 control DS/86 cells, or a total of 2×10^4 target cells consisting of 3) a 1:10 mixture of MRC/X and DS/86 cells, 4) a 1:2 mixture of MRC/X and DS/86 cells, 5) a 1:1 mixture of MRC/X and DS/86 cells, 6) a 2:1 mixture of MRC/X and DS/86 cells, or 7) a 10:1 mixture of MRC/X and DS/86 cells. A comparison of the [^{3}H]thymidine incorporation in the control reactions with the *trans*-costimulation reactions reveals a similar dose-response curve to that observed with CD80-mediated *trans*-costimulation of $CD8^+$ T lymphocytes. This experiment was repeated twice in triplicate, and the results were reproducible.

Discussion

Vaccination with tumor cells genetically modified to express CD80 or CD86 has been shown to induce protective immune responses in murine tumor model systems under certain conditions (4, 13–25, 37). These results underscore the potential of this strategy as an adjunct in the treatment of metastatic cancer and have formed the basis of a number of human clinical trials. However, as our results have clearly demonstrated, there are formidable logistic and technical challenges associated with genetically modifying primary human tumor tissue ex vivo that impede successful translation of the animal studies to human clinical investigation. Of the 41 primary human pediatric tumor samples we obtained, only one tumor grew sufficiently well in vitro to enable transduction with L86SN and G-418 selection of a CD86-expressing population to be successfully completed. Consequently, this was the only tumor sample that could be thoroughly tested for enhanced immunogenicity in an in vitro lymphocyte proliferation assay. Although the results of these in vitro assays validated the strategy, a success rate of ~3% for the gene transfer component of the procedure was problematic, and a reevaluation of the implementation strategy was required. In an effort o circumvent this limitation, we chose to investigate the possibility of using non-neoplastic bystander cells to provide the necessary costimulatory signal for T lymphocyte activation. Whereas the previously published murine studies (4, 13–25) relied upon the tumor cell providing both the Ag-specific signal and the costimulatory signal simultaneously (i.e., *cis*-costimulation), our strategy involved using a primary fibroblast transduced to express either CD80 or CD86 to provide the necessary costimulatory signal for $CD4^+$ or $CD8^+$ T lymphocyte activation in a *trans* configuration (i.e., *trans*-costimulation).

By nature, the *cis*-costimulation of T lymphocytes by target cells modified to express costimulatory signals would be expected to demonstrate one-hit kinetics, in that any target cell encountered by a T lymphocyte would provide both signal 1 (antigenic signal) and signal 2 (costimulatory signal) simultaneously. In contrast, *trans*-costimulation of T lymphocytes would be expected to demonstrate two-hit kinetics, in that the T lymphocyte would require contact with a cell providing signal 1 (antigenic signal) and a second cell providing signal 2 (costimulatory signal) to be activated. Thus, the relative efficiency of *cis*- and *trans*-costimulation can only be meaningfully compared under conditions that allow the inherently less favorable two-hit kinetics of *trans*-costimulation to take place. In short, the relative numbers of target cells providing signal 1 and signal 2 in the reaction must be kept high in relation to the effector

cells. Accordingly, reactions were configured to optimize *trans*-costimulation of T lymphocytes, and comparisons of efficiency with *cis*-costimulation were made under these conditions.

To test for *trans*-costimulation of $CD4^+$ T lymphocytes, we used allogeneic human fibroblasts pretreated with IFN-γ to induce MHC class II (HLA DP, DQ, DR) as a source of antigenic stimulation. When the purified $CD4^+$ T lymphocytes were incubated with these target cells, which could provide an Ag-dependent signal (signal 1) but no costimulation, the lymphocytes remained viable but no proliferation was detected. When a second population of allogeneic fibroblasts, which were MHC class II-negative but transduced to express CD80 or CD86 (signal 2), were added to the reaction the purified $CD4^+$ T lymphocytes were activated and lymphoproliferation occurred, as measured by [^{3}H]thymidine incorporation. These results clearly demonstrated that CD80- or CD86-mediated *trans*-costimulation of $CD4^+$ T lymphocytes could be achieved in an MHC class II-restricted reaction when 50% of the target cells provided signal 1 only and 50% provided signal 2 only. To determine the relative efficiency of this *trans*-costimulation reaction, a parallel series of experiments were performed in which the purified $CD4^+$ T lymphocytes were mixed with allogeneic MRC-5 fibroblasts that had either been transduced to express CD80 or CD86 and then treated with IFN-γ to simultaneously express MHC class II (i.e., *cis*-costimulation). The results of these comparative studies revealed that the efficiencies of CD86-mediated *cis*-costimulation and *trans*-costimulation of $CD4^+$ T lymphocytes in an MHC class II-restricted reaction were comparable under the defined conditions used in these reactions.

Having demonstrated CD80-mediated and CD86-mediated *trans*-costimulation of $CD4^+$ T lymphocytes, we then extended our studies to investigate the activation of $CD8^+$ T lymphocytes. By using a combination of allogeneic fibroblasts (MRC-5) to provide the Ag-specific signal (signal 1), and autologous fibroblasts (DS-fibroblasts) transduced to express either CD80 or CD86 to provide the *trans*-costimulation signal (signal 2), we were able to configure an assay that would test *trans*-costimulation of $CD8^+$ T lymphocytes in an MHC class I-restricted reaction. When the purified $CD8^+$ T lymphocytes were incubated with the allogeneic fibroblasts, or with autologous fibroblasts transduced to express either CD80 or CD86, they remained viable but no proliferation was detected. In contrast, when incubated with **a** 1:1 mixture of the allogeneic fibroblasts (signal 1) and the autologous fibroblasts expressing either CD80 or CD86 (signal 2), the $CD8^+$ T lymphocytes were activated and proliferation occurred, clearly demonstrating CD80-mediated and CD86-mediated *trans*-costimulation of $CD8^+$ T lymphocytes in an MHC class I-restricted lymphoproliferation assay. When the levels of proliferation resulting from *trans*-costimulation of $CD8^+$ T lymphocytes were compared with those achieved by a *cis*-costimulation reaction, in which the allogeneic fibroblasts (signal 1) were transduced to express either CD80 or CD86 (signal 2), the relative efficiencies of both reactions were comparable under the defined conditions used in the assay.

As mentioned earlier, a *cis*-costimulation reaction and a *trans*-costimulation reaction require different kinetics (one-hit and two-hit, respectively) to activate T lymphocyte proliferation, and as such both reactions can only be directly compared under the limited conditions discussed above. Moreover, one would predict that a *cis*-costimulation reaction could be detected at E:T ratios higher than those described in these assays, whereas the efficiency of a *trans*-costimulation reaction would be significantly diminished. This is because the probability of a T lymphocyte encountering both a target cell expressing signal 1 and a second target cell expressing signal 2 would decrease more rapidly in a *trans*-costimulation reaction than would occur when the target cell expressed both signals simultaneously (*cis*-costimulation). In other words, the relative efficiency of a *cis*-costimulation reaction at an E:T ratio higher than 10:1 (e.g., 100:1) would be expected to reduce as a direct function of the dilution factor, whereas the efficiency of a *trans*-costimulation reaction would, as a first approximation, be expected to reduce as a function of the square of the dilution factor. Nonetheless, we were interested in 1) determining the effect of varying the ratio of target cells expressing signal 1 to target cells expressing signal 2 in a *trans*-costimulation reaction of $CD4^+$ and $CD8^+$ T lymphocytes and 2) comparing the *trans*-costimulatory capacity of CD80 with CD86 under these conditions. The results of these studies revealed that *trans*-costimulation of $CD8^+$ and $CD4^+$ T lymphocytes (in MHC class I- and class II-restricted reactions, respectively) was detectable in dose-response reactions in which the ratio of target cells expressing signal 1 to target cells expressing signal 2 was varied from 10:1 to 1:10. In addition, the magnitude of the lymphoproliferation, as measured by [^{3}H]thymidine incorporation, mediated by CD80 or CD86-transduced target cells was comparable at each of the defined ratios.

In terms of therapeutic and biological implications, the issue of differential relative efficiencies for *cis*-costimulation and *trans*-costimulation reactions as a function of the E:T ratio is unlikely to be problematic. For example, in a cancer immunotherapy strategy, *trans*-costimulation of anti-tumor immune response against a potentially immunogenic tumor could be attempted using a patient's own fibroblasts to provide the CD80- or CD86-mediated costimulatory signal (signal 2). Given that signal 1 (expressed by the tumor cell) would be constantly in excess and that we have shown *trans*-costimulation will occur if a few as 10% of the bystander cells are expressing signal 2, an appropriate in situ inoculum of CD80- or CD86-modified autologous fibroblasts should be capable of providing sufficient costimulation to any tumor-reactive T lymphocytes that have received signal 1. In vivo studies using murine tumor model systems are currently underway to directly address this question. Moreover, the results presented here also suggest a number of intriguing possibilities for links between *trans*-costimulation of T lymphocytes in vivo and the etiology of autoimmune disease. This possibility was discussed by Liu and Janeway, who, on the basis of their in vitro data, suggested that *trans*-costimulation would be inefficient and unlikely to induce an autoimmune response (28). More recently, Ding and Shevach described efficient *trans*-costimulation of murine $CD4^+$ T lymphocytes by transfected L cells expressing CD80 and suggested the possibility that *trans*-costimulation of naive T lymphocytes could be induced in vivo (31). However, they noted that the levels of CD80 expression required for *trans*-costimulation to occur were much higher than found on the surface of any normal APC, and therefore CD80-mediated *trans*-costimulation of autoreactive T lymphocytes was unlikely to be a problem under normal physiological conditions. However, our results suggest that this may not be the case, at least for CD86-mediated *trans*-costimulation. In addition to demonstrating that CD86-mediated *trans*-costimulation is an efficient means of activating both $CD4^+$ and $CD8^+$ T lymphocytes, the levels of CD86 expression required for the process are equivalent to those that we have previously characterized on the surface of peripheral blood monocytes and PBMC-derived dendritic cells, two APC that constitutively expresses this costimulatory molecule (34, 38). Therefore, it is at least theoretically possible that an APC, such as a peripheral blood monocyte, could *trans*-costimulate an autoreactive T lymphocyte in vivo, and because autoimmune disease is a relatively rare clinical problem, there may be other as yet unidentified mechanisms regulating and/or inhibiting this reaction. Additional studies will be required to resolve this question.

Interestingly, the absolute level of [^{3}H]thymidine uptake observed with either *cis*-costimulation or *trans*-costimulation of $CD4^+$ T lymphocytes was consistently ~10-fold higher than that observed with $CD8^+$ T lymphocytes. The most likely explanations for this difference are 1) that activated $CD4^+$ T lymphocytes produce IL-2 (whereas $CD8^+$ T lymphocytes do not), which can have autocrine stimulatory activities, and 2) that only ~50% of human peripheral blood $CD8^+$ T lymphocytes express CD28, the primary counter-receptor for CD80 and CD86, and therefore the $CD28^-$ T lymphocytes are unlikely to be activated in these assays. Further in vitro studies are planned to address these possibilities. Nonetheless, these studies have established that *trans*-costimulation can be an efficient activator of human $CD8^+$ and $CD4^+$ T lymphocytes in MHC class I- and class II-restricted allogeneic reactions, respectively.

Although an in vitro lymphoproliferation assay in only one measure of T lymphocyte activation, we chose to initially examine *trans*-costimulation by this established and well-validated methodology to enable a direct comparison with the existing literature on this subject. Of the four published reports discussing the efficiency of *trans*-costimulation in the process of T lymphocyte activation (26, 28, 30, 31), only one directly examined CD86-mediated *trans*-costimulation. In this report, *trans*-costimulation was demonstrated to be between 10- and 30-fold less efficient than *cis*-costimulation of T lymphocytes (26). Given the differences between our two assay systems in this instance, one possible explanation for the higher efficiency of *trans*-costimulation in our studies may be the use of human fibroblasts to provide the CD86-mediated costimulatory signal. In the previous study, CHO cells expressing CD86 were used to costimulate the $CD4^+$ T lymphocytes, and it is conceivable that the use of xenogeneic cells did not provide the necessary species-specific ICAMS and/or other minor costimulatory molecules required for efficient T lymphocyte activation. The remaining three publications discuss CD80-mediated *trans*-costimulation of $CD4^+$ T lymphocytes, with differing conclusions (28, 30, 31). In these studies, *trans*-costimulation was reported to range from 80-fold less efficient than *cis*-costimulation (if it occurred at all) to equally as efficient as *cis*-costimulation in the activation of $CD4^+$ T lymphocytes (28, 30, 31). In contrast, the results of our studies reveal that both CD80 and CD86 are efficient mediators of *cis*-costimulation and *trans*-costimulation of human $CD4^+$ T lymphocytes in an MHC class II-restricted reaction. Importantly, we have established that *trans*-costimulation works with $CD8^+$ T lymphocytes, which remain a key effector cell in any tumor immunotherapy strategy.

Overall, the demonstration that CD80- and CD86-mediated *trans*-costimulation of $CD4^+$ and $CD8^+$ T lymphocytes is efficient in vitro is a significant first step toward exploiting this process therapeutically. Clearly, additional studies will be necessary to compare and contrast *cis*-costimulation with *trans*-costimulation to determine which cytokines are produced by the activated $CD4^+$ and $CD8^+$ T lymphocytes, whether or not the T lymphocytes are preferentially driven down a Th1 or Th2 pathway, whether or not fully matured CTL can be generated, and what if any differences result from T lymphocyte activation by CD80-mediated *cis*- or *trans*-costimulation when compared with CD86-mediated reactions. Nonetheless, the demonstration of efficient *trans*-costimulation of $CD4^+$ and $CD8^+$ T lymphocytes in vitro provides an alternative strategy for further investigation in efforts to develop an immune augmentation cancer therapy. Not only would the strategy of using a patients' own fibroblasts to provide the necessary costimulatory signal obviate many of the technical problems associated with ex vivo manipulation of primary tumor tissue, but it may also enable the procedure to be applied to tumors resistant to gene transfer by current technologies. Finally, if *trans*-costimulation can be used to generate functionally matured effector T lymphocytes, it may be possible to selectively modulate humoral and cell-mediated immune responses in an effort to develop the most effective anti-tumor therapy for each type of malignancy, which may be particularly relevant to treatment of tumors that have down-regulated MHC class I expression.

Acknowledgments

We thank Christine Smyth for excellent technical assistance with the immunofluorescence confocal microscopic studies and the FACScan analyses and Drs. Luciano Dalla-Pozza, Susan Arbuckle, Claire Cooke-Yarborough, and Ms. Gabriella Palmer for collecting, classifying, and supplying primary pediatric tumor samples.

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