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*J Immunol* 2000; 165:1925-1932; doi: 10.4049/jimmunol.165.4.1925

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Regulation of Gene Expression of Murine MD-1 Regulates Subsequent T Cell Activation and Cytokine Production

Reginald M. Gorczynski,1 Zhigi Chen, David A. Clark, Jiang Hu, Gary Yu, Xiarong Li, Wendy Tsang, and Sima Hadidi

The immunoadhesin (OX2:Fc) comprising the extracellular domain of murine OX2 linked to IgG2aFc, inhibits production of IL-2 and IFN-γ by activated T cells and increases allograft and xenograft survival in vivo. Increased expression of OX2 on dendritic cells (DC) in vivo following preimmunization via the portal vein is also associated with elevated expression of MD-1. We have used antisense oligodeoxynucleotides (ODNs) to MD-1 to investigate the effect of inhibition of expression of MD-1 by DC on their function as allosstimulatory cells. We also investigated by FACS analysis the cell surface expression of OX2, CD80, and CD86 on DC incubated with ODN-1 blocking MD-1 expression. Blocking MD-1 gene expression inhibits surface expression of CD80 and CD86, but not of OX2. DC incubated with ODN-1 to MD-1 did not stimulate IL-2 or IFN-γ production, but generated cells able to suppress, in a second culture of fresh DC plus allogeneic T cells, production of IL-2 and IFN-γ. This inhibition was blocked by anti-OX2 mAb. Infusion of DC preincubated with ODN-1 prolonged renal allograft survival, an effect also reversed by anti-OX2 mAb. By FACS, incubation of DC with anti-MD-1 Ab to promote capping eliminated cell surface expression of MD-1 and CD14 anti-OX2 mAb. Infusion of DC preincubated with ODN-1 prolonged renal allograft survival, an effect also reversed by anti-OX2 mAb. Thus OX2 and MD-1 are independent surface molecules on DC that may reciprocally regulate T cell stimulation. MD-1 is linked to CD14, a “danger receptor complex,” and activation of this complex can regulate cell surface expression of CD80/CD86, which signal T cells.

A T cell is activated after engagement of its TCR by Ag presented on APC in association with MHC molecules and delivery of costimulatory signals resulting from the interaction of several ligand/coreceptor complexes (1–3). Cytokines provided by the APC and other T cells reacting to Ag appear necessary for full development and maturation of the response. The primary “positive” costimulatory interactions are provided by engagement of CD40 ligand with CD40 and CD28 with CD80/CD86. The function of APC in inducing immune responses depends on the regulated expression of both cytokines (which act on T cells) and costimulatory molecules. Because it is known that the expression of costimulatory molecules on APC is itself often altered by exposure of APC to local cytokines (4), costimulation may be regulated by a local bidirectional cytokine-mediated signaling between a T cell and its APC. For example, CD40 ligand expression on Ag-activated T cells interacting with CD40 on the APC may generate a signal to the APC that augments CD80/CD86 expression on the APC; the T cell is then stimulated further by CD80/CD86 interacting with CD28. Such a model helps explain the synergistic inhibition of graft rejection by blocking both CD40 and CD28 (3). Despite the importance of these costimulatory pathways in T cell triggering, inhibition of T cell stimulation has not reproducibly been seen by blocking costimulation alone. Some receptors on T cells, such as CTLA4, are thought to convey an inhibitory signal to the T cell following engagement by CD80/CD86. In support of this idea, injection of CTLA4Ig has been shown to prolong graft survival in vivo (5–9). However, this positive effect on graft survival has not been seen consistently using CTLA4Ig (10–12).

In a mouse model system, allograft survival is increased following donor-specific portal vein (pv)3 immunization (13, 14). Using a DNA subtractive hybridization approach, we showed that immunoregulation in pv-immunized mice is associated with increased expression of a number of distinct mRNAs in the liver (15). One was shown to encode OX2, a molecule expressed on the surface of dendritic cells (DC). OX2 was initially described by Barclay (16) as a molecule with unknown function, expressed in the thymus, brain, and uterus. Detailed examination of OX2 expression, constitutive and/or inducible, on cells other than DC (and macrophages; R. M. Gorczynski, unpublished observation) has not been reported. We showed that anti-OX2 mAbs blocked the protective effect of pv immunization in mice receiving renal allografts (15) and rats receiving small intestinal transplants (17). Anti-OX2 also blocked the polarization of T cells to produce type 2 cytokine production induced by pv immunization (15, 17). More recently, we reported on a soluble immunoadhesin, OX2:Fc, in which the extracellular domain of OX2 was genetically linked to a murine IgG2aFc region (with mutations in the FcR and C1q binding regions to impair binding functions). OX2:Fc inhibited T cell allostimulation and type 1 cytokine production (IL-2, IFN-γ) in vitro and in vivo (18) and prolonged survival of allografts and xenografts in vivo (19). These and other data (19) support the hypothesis that OX2 is a novel “coregulatory” molecule, which controls the functional outcome of the TCR:Ag encounter.

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2 Abbreviations used in this paper: pv, portal vein; DC, dendritic cells; ODN, oligodeoxynucleotide; PRR, pattern recognition receptor; aF10, α-MEM supplemented with 2-ME and 10% FBS; LRR, leucine-rich repeat; CHO, Chinese hamster ovary.
In addition to OX2, we also described other molecules that were differentially expressed following PV immunization (15). The full-length sequence for one of these (clone 71 in Ref. 15) was recently identified as the murine homologue of chicken MD-1 (20). MD-1 has been reported to regulate expression of RP105 in B cells (21). RP105, which is also expressed on DC (22), is a member of a family of molecules bearing a leucine-rich repeat (LRR) motif that serves an important, and evolutionarily conserved, function in immunity in a number of species (23). This family, which includes CD14, a ligand for LPS (24), comprises receptors for invariant molecular structures in pathogens (pattern recognition receptors, PRRs) that trigger innate cellular immune responses to “danger.” PRR triggering leads to the induction of proinflammatory cytokines (IL-1, IL-8, IL-6, IFN-γ) and up-regulation of certain co-stimulatory molecules (e.g., CD80) (25), effects that might be anticipated to promote a rejection response.

Based on these observations, we hypothesized that, following PV immunization, there occurs an imbalance in regulation of expression of prorejection costimulants, such as CD80 and/or CD86, under the influence of MD-1, and prograft-maintaining signals, such as OX2. This hypothesis was tested in the studies below, using DC preincubated with antisense oligodeoxynucleotides (ODNs) to MD-1. We examined expression of mRNAs (by PCR) for CD80, CD86, OX2, and MD-1 as well as the surface expression of those same molecules by FACS. In addition, we have examined the effect of these ODNs on the stimulation of cytokine production in allogeneic T cells by treated DC, as well as the ability of those DC to induce increased renal allograft survival. The data appear to support the hypothesis.

Materials and Methods

Mice

Male C57HHeJ, BALB/c, and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed five per cage and allowed food and water ad libitum. All mice were used at 8–12 wk of age. Fischer 344 (Fi) rats purchased from Harlan Sprague Dawley (Indianapolis, IN) were used for immunization to produce monoclonal anti-MD-1 Abs.

Monoclonal Abs and cytokines

The following mAbs were obtained from Pharmingen (San Diego, CA): anti-IL-10 (JES5-2A5; biotinylated, SXC-1), anti-IL-2 (S4B6; American Type Culture Collection, Manassas, VA), anti-IL-4 (1B11; American Type Culture Collection), anti-IFN-γ (R4-6A2; American Type Culture Collection; biotinylated XMG1.2), FITC-anti-CD80, FITC-anti-CD86, FITC-anti-CD40, and anti-CD4, FITC-anti-CD4, and anti-CD8, rabbit complement, FITC-anti-CD3, and FITC-MAC-1 were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). DEC205 (anti-mouse DC) and F(ab′)2 rabbit anti-rat IgG FITC conjugate (nonreactive with mouse IgG) were obtained from Serotec (Mississauga, Ontario, Canada). Rat anti-mouse OX2 (3B6) was purchased from BioSpark (Mississauga, Ontario, Canada) (26). Streptavidin HRP and recombinant mouse GM-CSF were purchased from Pharmingen.

Preparation of cells

Single-cell spleen suspensions were prepared aseptically from individual mice in each experiment. After centrifugation, the cells were resuspended in α-MEM supplemented with 2-ME and 10% FBS (αF10). Bone marrow-derived DC were obtained by culture of bone marrow cells in vitro (27). Bone marrow cells were pooled from three donors, treated with the mixture of Abs (L3T4, anti-thy1.2, anti-Ly2.2) and rabbit complement, and 2 × 10⁶ cells were cultured in 10 ml of αF10 in tissue culture flasks with 500 U/ml recombinant murine GM-CSF (PharMingen). Fresh GM-CSF was added every 36 h. Cells were separated over Lymphopaque (Cedarlane Laboratories) on days 3, 5, and 7 of culture and recultured in αF10 with recombinant GM-CSF. An aliquot of the cells was stained at 10 days with DEC205 and FITC anti-rat IgG or, as control, with FITC-anti-CD3. Staining with these Abs averaged 96 ± 6 and <4%, respectively. The remaining cells were washed, counted, and used as described below.

Stimulation with LPS (from Brucella abortus, a kind gift from Dr. C. Galanos, Max Planck Institute for Immunobiology, Freiburg, Germany), was performed for 18 h (see text), using LPS at a concentration of 250 ng/ml.

PV immunization

PV immunization was performed as described earlier (28). The mice were anesthetized with Nembutal (MTC Pharmaceuticals, Cambridge, Ontario, Canada), and 1 × 10⁶ of the cultured allogeneic DC were injected in 0.1 ml through a superior mesenteric vein using a 30-gauge needle. After injection, the needle was rapidly withdrawn and hemostasis was secured by gentle pressure with 2-mm gel foam. Complications (hemorrhage postinjection) were seen in <10% of mice, and these were excluded from analysis.

Renal transplantation

This procedure was performed as described elsewhere (14). In brief, one kidney was removed and replaced with the donor kidney, with the remaining host kidney excised 2 days later. All renal transplant recipients received an i.m. injection of cefotetan (30 mg/kg) on the day of transplantation and for 2 succeeding days. All animals received i.m. cyclosporin A (15 mg/kg) daily for the first 2 days posttransplant. Pretreatment for the first 2 days posttransplant. Pretreatment treatment with mAb 3B6 following transplantation, five sequential i.v. injections of 100 µg Ig in 300 µl saline were given 36 h apart, beginning at the day of transplantation. Control animals received equivalent injections of a control Ig (from pooled normal rat serum).

Cytotoxicity and cytokine assays

To assess induction of CTL and/or cytokine production, C3H/HeJ responder cells were stimulated with an equal number of mitomycin-C-treated (45 min at 37°C) allogeneic or control spleen stimulator cells in triplicate cultures in αF10. Supernatants were pooled at 40 h from replicate wells and each was assayed in triplicate in ELISA for cytokine production as described below. As discussed elsewhere (19), we have routinely observed no difference in cytokine production over the period from 36 h to 54 h in cultures stimulated with allogeneic DC. In some experiments, the cultures were continued to 72 h and received 1 µCi/well of [³¹]Cr-B-h recombining splenocyte blasts as target cells. Supernatants were sampled at 4 h from stimulation of the percentage of specific [³¹]Cr release.

IL-2 and IL-4 activity were assayed by bioassay using the IL-2 or IL-4-dependent cell lines, CTL-L2 and CTT-S, respectively. Recombinant cytokines for standardization of assays was purchased from Genzyme (Cambridge, MA). IL-2 assays were set up in the presence of 11B11 Ab to block potential stimulation of CTL-L2 by IL-4; IL-4 assays were set up in the presence of S4B6 Ab to block IL-2-mediated stimulation. Both the IL-2 and IL-4 assays reproducibly detected 20 pg of recombinant cytokine added to cultures. The concentration of mAbs used blocked 50 ng of cytokine activity. IFN-γ and IL-10 were assayed using ELISA. For IFN-γ, the assay used flat-bottom 96-well Nunc plates (Life Technologies, Rockville, MD) coated with 100 ng ml⁻¹ R4-6A2. Varying volumes of supernatant were bound in triplicate at 4°C and washed three times, and biotinylated anti-INF-γ (XMG1.2) was added. After washing, the plates were incubated with streptavidin-HRP (Cedarlane Laboratories), developed with an appropriate substrate, and OD₄₅₀ was determined using an ELISA plate reader. Recombinant IFN-γ for standardization was obtained from Pharmingen. IL-10 was assayed using a similar ELISA system with JES5-2A5 as the capture Ab and biotinylated SX-1 as developing Ab. Recombinant IL-10 for standardization of this assay was obtained from PeproTech (Rocky Hill, NJ). Each assay reliably detected the relevant cytokine at levels in the range 40–4000 pg/ml.

Ag preparation, immunization, and production of mAb to MD-1

Details on the preparation and characterization of these reagents is given elsewhere (S. Hadidi J. Lei, and R. M. Gorczynski, manuscript in preparation). In brief, two Fisher rats were immunized by i.p. injections of 200 µg of keyhole limpet hemocyanin-coupled peptide (representing an exposed epitope for the predicted amino acid sequence of MD-1 as determined by hydrophobicity plots). Following boosting, spleen cells were harvested, pooled, and used for fusion with YR2/2 parental myeloma cells as previously described (29). One-step selection and cloning of the hybridomas was performed in 0.8% methylcellulose medium (Immuno-Precise
Antibodies, Victoria, British Columbia, Canada), as described in detail elsewhere (26). Clones were picked and resuspended in wells of 96-well tissue culture plates in 200 μl of α-MEM containing 1% hyposoxanthine/ thymidine, 20% FBS, 1% OPL, and 1 × 10^6 BALB/c thymocytes/ml, and culture supernatants were screened by FACS for detection of MD-1 as described elsewhere using Chinese hamster ovary (CHO) cells transduced to express murine MD-1 (see also Ref. 26). The mAb SH1.2.47 detected a molecule in extracts of DC with molecular size of 27–30 kDa (the reported size of murine MD-1 is 28 kDa) (30), and stained CHO cells transduced with adenoval vectors engineered to contain a single copy of MD-1 cDNA, as per published sequence, inserted into the NotI/ BamHI sites. Control CHO cells were transduced with vector containing no MD-1 construct and showed only background staining. FITC anti-rat IgG was used as secondary Ab.

Preparation and testing of antisense ODNs for MD-1

A series of antisense ODNs to MD-1 were prepared by Midland Reagent (Midland, TX) as described (31) using phosphorothioate modification to produce nuclease-resistant material. After preliminary testing to delineate active compounds, the final ODNs used were prepared using further C-5 propynyl modification of pyrimidines in the phosphorothioate starting material (32). Delivery of ODNs to DC in culture used the cationic lipid GS2888 cytofectin (32). In control studies with other cDNAs, we have routinely observed ~40% transduction efficiency with this technique (33). Following 6 h of incubation in serum and antibiotic free α-MEM, the cells were incubated overnight in α-F10 with 250 μg/ml LPS. Then, the cells were washed exhaustively (four times) with α-F10 and used in the assays described. The sequence of the ODNs used is shown below. The location of these sequences in the coding sequence of murine MD-1 (nt 95–583, as per Miyake et al. (30)) is shown in parenthesis: ODN-1, 5′-AG GGCACGUCGGCAACC-3′ (nt 119–95); ODN-2, 5′-CCUGUGGAAACAU CAAGU-3′ (nt 265–241); ODN-3, 5′-AGGGAACCUUGGUGCCUC-3′ (nt 583–559).

Analysis of inhibition of expression of a control gene (GAPDH) and MD-1, OX2, CD80, and CD86, using ODNs to MD-1, was performed by PCR and Northern analysis in cells transduced with antisense ODNs. FACS analysis using FITC-labeled anti-CD80, anti-CD86, OX2, and DEC205 mAbs was performed on the ODN-treated cells. In addition, they were used at varying numbers (from 1 × 10^4 to 3 × 10^5) as stimulator cells (following mitomycin C treatment) for allogeneic C3H/HeJ mouse spleen cells transduced with adenoviral vectors engineered to contain a single copy of MD-1 cDNA, as per published sequence, inserted into the NotI/ BamHI sites. Control CHO cells were transduced with vector containing no MD-1 construct and showed only background staining. FITC anti-rat IgG was used as secondary Ab.

Preinoculation of DC before LPS addition

FIGURE 1. Antisense ODNs to MD-1 inhibits expression of MD-1 mRNA, as well as CD80/CD86 mRNA, as determined by comparative densitometry in Northern gels (mean of three experiments); see Materials and Methods for more details. RNA was extracted from bone marrow-derived DC after overnight LPS stimulation of DC that had been incubated for 6 h with medium alone, cytofectin, or cytofectin with 20 nM of the ODNs shown. Control studies (not shown) in which 1–100 nM of ODNs were used indicated that optimal specific inhibition of MD-1 expression, without affect on GAPDH expression, was seen using 20 nM of ODNs. Data show mean (± SD) relative to a medium control GAPDH set as 1.

Results

Inhibition of mRNA by ODNs and surface expression of MD-1, CD80/CD86, and OX2

In our first series of experiments, we asked whether antisense ODNs to MD-1 would specifically inhibit DC transcription/translation and surface expression of MD-1 without altering expression of a number of other molecules important in T cell stimulation, such as CD80, CD86, and OX2. DC were derived from 10-day bone marrow cultures and transduced with ODNs as described in Materials and Methods. mRNA expression (assayed by PCR) of GAPDH was used as one control, whereas in cells tested by FACS analysis, we assessed the expression of DEC205 as a control Ag. Fig. 1 shows results, averaged over three studies, where ODN-1

Inhibition by ODNs of cell surface expression of molecules on DC

FIGURE 2. FACS analysis of cell surface expression of DC treated as in Fig. 1, but then stained with the various mAbs shown (rather than used for RNA extraction). FITC-labeled CD80 and CD86 were used; no staining was seen with FITC-labeled isotype controls (data not shown). FITC staining with other mAbs used an indirect reaction with FITC-anti-rat Ig (staining with FITC anti-rat Ig alone is shown as a solid curve in a, b, and e).
treatment inhibited mRNA expression of MD-1, but not of GAPDH, as assayed by densitometry comparisons of Northern gels, using oligonucleotide probes for the various mRNAs synthesized according to published sequences. There was some inhibition of CD80 and CD86 mRNA, though no perturbation of OX2 mRNA expression was detectable. The inhibition of CD80/CD86 mRNA expression in LPS-stimulated cells following treatment with ODNs to MD-1 presumably represents an indirect effect mediated by down-regulation of the LPS stimulation (by NF-κB) of induction of these mRNAs following blockade of MD-1 signaling (21, 25).

Fig. 2 shows a typical result (from one of six studies) using FACs analysis. Clear evidence for inhibition of surface expression of MD-1 and CD80, CD86 was seen following treatment with ODN-1 and -2, but not with ODN-3. No significant inhibition of surface expression of OX2 or DEC205 was seen by FACs analysis. In addition, in other studies (not shown) no modification of cell surface expression of class II MHC was seen following any of the treatments shown. No investigations have been performed to assess effects on class I MHC expression. In all cases, no inhibition of MD-1 and CD80, CD86 was seen following treatment with anti-MD-1 (and anti-rat Ig) at 37°C simultaneously reduces subsequent staining of LPS-stimulated DC with both MD-1 and CD14, but not with anti-CD80, anti-CD86, anti-OX2, or the control anti-DC Abs, DEC205, or DC26. Cells in the columns shown were preincubated (60 min at 4°C) in medium, rat anti-DC26, or anti-MD-1 (SH1.2.47), before washing and incubation at 37°C with anti-rat Ig. Thereafter, cells were stained with different mAbs as in Fig. 2.

FIGURE 3. Pretreatment with anti-MD-1 (and anti-rat Ig) at 37°C simultaneously reduces subsequent staining of LPS-stimulated DC with both MD-1 and CD14, but not with anti-CD80, anti-CD86, anti-OX2, or the control anti-DC Abs, DEC205, or DC26. Cells in the columns shown were preincubated (60 min at 4°C) in medium, rat anti-DC26, or anti-MD-1 (SH1.2.47), before washing and incubation at 37°C with anti-rat Ig. Thereafter, cells were stained with different mAbs as in Fig. 2.

Table I. DC incubated with antisense ODNs to MD-1 fail to stimulate proliferation or induction of CTL/type 1 cytokines in allogeneic responder cells

<table>
<thead>
<tr>
<th>Added ODNs</th>
<th>[3H]TdR (cpm)</th>
<th>Percent Lysis</th>
<th>Cytokines in Culture (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(50:1, E:T)</td>
<td>[51Cr] Targets</td>
<td>IL-2</td>
</tr>
<tr>
<td>None</td>
<td>9920 ± 1400</td>
<td>44 ± 5.7</td>
<td>1080 ± 190</td>
</tr>
<tr>
<td>ODN-1</td>
<td>1220 ± 190*</td>
<td>6.4 ± 2.2*</td>
<td>180 ± 60*</td>
</tr>
<tr>
<td>ODN-2</td>
<td>5275 ± 960</td>
<td>24 ± 5.3</td>
<td>685 ± 135</td>
</tr>
<tr>
<td>ODN-3</td>
<td>8550 ± 1350</td>
<td>38 ± 6.1</td>
<td>1135 ± 175</td>
</tr>
</tbody>
</table>

* C57BL/6 bone marrow-derived DCs were incubated with medium alone (control-first row) or with antisense ODNs to MD-1 (see Materials and Methods and Fig. 1–3). After further overnight stimulation with LPS, cells were treated with mitomycin C and used as stimulators in triplicate cultures (3:1, responder:stimulator ratio) for C3H spleen cells (pooled from four mice). Cultures were harvested and examined for proliferation, CTL production, or cytokine production. Without LPS stimulation, all DC induced significantly less proliferation, CTL, and IL-2 production. Thus from untreated DC (no LPS), these values were 2460 ± 290, 11 ± 2.9, and 275 ± 65, respectively.

Cytokines (pg/ml) in culture supernatants harvested at 40 h.

Cytokines (pg/ml) in culture supernatants harvested at 40 h.

* p < 0.05 compared with control in first row.
Table II. Titration of allostimulation in vitro, using DC incubated with antisense ODNs to MD-1

<table>
<thead>
<tr>
<th>Added ODNs</th>
<th>Stimulator Ratio</th>
<th>[3H]Tdr (cpm)</th>
<th>Percent Lysis [%Cr Targets (50:1, E:T)]</th>
<th>IL-2 in Culture (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10:1</td>
<td>6750 ± 1050</td>
<td>34 ± 6.2</td>
<td>610 ± 135</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>9560 ± 1250</td>
<td>47 ± 6.1</td>
<td>1130 ± 170</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>8765 ± 1070</td>
<td>45 ± 5.4</td>
<td>1035 ± 195</td>
</tr>
<tr>
<td>ODN-1</td>
<td>10:1</td>
<td>1020 ± 210*</td>
<td>3.1 ± 1.9*</td>
<td>150 ± 55*</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>1230 ± 195*</td>
<td>4.3 ± 2.1*</td>
<td>180 ± 60*</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>1630 ± 180*</td>
<td>5.4 ± 2.2*</td>
<td>190 ± 65*</td>
</tr>
<tr>
<td>ODN-3</td>
<td>10:1</td>
<td>5970 ± 970</td>
<td>33 ± 5.3</td>
<td>680 ± 150</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>9180 ± 1105</td>
<td>45 ± 5.9</td>
<td>1010 ± 210</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>8990 ± 1185</td>
<td>47 ± 6.3</td>
<td>1105 ± 190</td>
</tr>
</tbody>
</table>

*–d As for Table I. Untreated and ODN-treated LPS-stimulated DC were used at various responder:stimulator ratios in triplicate cultures, as shown.

*, p < 0.05 compared with control cultures (untreated DC, same responder:stimulator ratio).

DC incubated with antisense ODNs for MD-1 do not stimulate type 1 cytokine production

Because it is believed that CD80 and/or CD86 provide an important costimulatory signal for type 1 cytokine production by allostimulated T cells, we next asked whether DC incubated with ODNs to block MD-1 and CD80/CD86 expression would be impaired in this ability. Bone marrow-derived DC of C57BL/6 origin were incubated with ODNs 1–3, cultured overnight with LPS, treated with mitomycin C, and used as stimulator cells for cultures containing allogeneic C3H spleen responder cells. Proliferation and cytokine production in these cultures was assayed. In addition, separate cultures were incubated for 5 days for analysis of CTL. Data for one of four such studies are shown in Table I.

Incubation of DC with ODN-3 produced no significant effect on the allostimulatory properties of the DC (see first and last rows of Table I), in keeping with the lack of effect of this antisense ODN on expression of costimulatory molecules (see Figs. 1 and 2). In marked contrast, DC incubated with ODN-1, which inhibits expression of MD-1 and CD80/CD86 at the cell surface after further LPS stimulation of ODN-treated DC (see Fig. 2), were no longer capable of stimulation of CTL, proliferation, or type 1 cytokine production by the allogeneic spleen responder cells (see Table I). There was a trend to increased production of IL-4 and IL-10 in response to ODN-1-treated DC. Data using DC incubated with ODN-2 was intermediate between that observed with ODN-1- and ODN-3-treated cells, as anticipated from the data of Fig. 1. Failure to stimulate with ODN-1/2-treated DC was not overcome by increased DC in vitro (Table II). In the absence of overnight LPS stimulation, all DC were much less efficient at allostimulation (see Table I). Accordingly, and because the effect of ODN-1 treatment on CD80/CD86 expression on DC was observed after LPS stimulation (see Fig. 2), results from this experiment are reported only for LPS-stimulated DC.

Table III. DC incubated with antisense ODNs to MD-1 induce cells able to suppress Ag-specific proliferation or induction of CTL type 1 cytokines in allogeneic responder cells

<table>
<thead>
<tr>
<th>Added ODNs</th>
<th>[3H]Tdr (cpm)</th>
<th>Percent Lysis [%Cr Targets (50:1, E:T)]</th>
<th>Cytokines in Culture (pg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-2</td>
<td>IL-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-10</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Secondary cultures stimulated with C57BL/6 DC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8220 ± 1150</td>
<td>38 ± 4.8</td>
<td>1120 ± 210</td>
</tr>
<tr>
<td>None</td>
<td>9200 ± 1250</td>
<td>35 ± 5.2</td>
<td>1010 ± 165</td>
</tr>
<tr>
<td>ODN-1</td>
<td>2820 ± 550*</td>
<td>12 ± 4.2*</td>
<td>290 ± 90*</td>
</tr>
<tr>
<td>ODN-3</td>
<td>8900 ± 1405</td>
<td>35 ± 6.0</td>
<td>1065 ± 225</td>
</tr>
<tr>
<td>Secondary cultures stimulated with BALB/c DC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9100 ± 1210</td>
<td>40 ± 5.1</td>
<td>1010 ± 230</td>
</tr>
<tr>
<td>None</td>
<td>9720 ± 1550</td>
<td>42 ± 6.0</td>
<td>1230 ± 210</td>
</tr>
<tr>
<td>ODN-1</td>
<td>8200 ± 1190</td>
<td>44 ± 5.9</td>
<td>980 ± 160</td>
</tr>
<tr>
<td>ODN-3</td>
<td>8980 ± 1050</td>
<td>39 ± 5.7</td>
<td>1035 ± 195</td>
</tr>
</tbody>
</table>

* C57BL/6 bone marrow-derived DCs were incubated with medium alone (None) or with one of two different antisense ODNs to MD-1 (see Materials and Methods and Table I). After further overnight stimulation with LPS, cells were washed thoroughly, treated with mitomycin C, and used as stimulators in triplicate cultures (3:1 responder:stimulator ratio) for C3H spleen cells (pooled from four stock mice). Cells were harvested at 5 days, washed, and counted. A total of 2 × 10^5 cells were added in triplicate to secondary cultures containing 5 × 10^5 C3H responder cells and 2 × 10^6 fresh C57BL/6 or BALB/c DC. Control cultures received no added cells from the first culture. There were no detectable surviving mitomycin C-treated C57BL/6 cells (as determined by FACS analysis with FITC-anti-H2K^b^) transferred from the primary cultures into secondary cultures in these studies; limit of FACS analysis ~0.5%.

*, p < 0.05 compared with control in first row.
Table IV. Anti-OX2 mAb inhibits suppression mediated by DC incubated with antisense ODNs to MD-1

<table>
<thead>
<tr>
<th>Added ODNs*</th>
<th>[3H]Tdr (cpm)*</th>
<th>Percent Lysis of 51Cr Targets (50:1, E:T)*</th>
<th>Cytokines in Culture (pg/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td>Control Ab added to cultures receiving ODN-treated DC</td>
<td>9520 ± 950</td>
<td>31 ± 3.9</td>
<td>1210 ± 190</td>
</tr>
<tr>
<td>None</td>
<td>650 ± 1050</td>
<td>32 ± 4.6</td>
<td>1150 ± 190</td>
</tr>
<tr>
<td>ODN-1</td>
<td>2350 ± 690*</td>
<td>9.2 ± 3.6*</td>
<td>330 ± 80*</td>
</tr>
<tr>
<td>ODN-3</td>
<td>9490 ± 1285</td>
<td>30 ± 5.6</td>
<td>1220 ± 220</td>
</tr>
</tbody>
</table>

Anti-OX2 mAb added to cultures receiving ODN-treated DC

| Control     | 8970 ± 1340 | 33 ± 5.3                         | 1320 ± 210 | 55 ± 15 | 950 ± 140 | 50 ± 15 |
| None        | 9155 ± 1350 | 34 ± 4.6                         | 1195 ± 160 | 50 ± 15 | 990 ± 175 | 55 ± 10 |
| ODN-1       | 7530 ± 1560 | 25 ± 4.8                         | 885 ± 230  | 85 ± 25 | 690 ± 185 | 75 ± 25 |
| ODN-3       | 8360 ± 1285 | 31 ± 5.2                         | 1100 ± 160 | 65 ± 20 | 825 ± 175 | 50 ± 15 |

* C57BL/6 bone marrow-derived DCs were incubated with medium alone (None) or with one of two different antisense ODNs to MD-1 (see Materials and Methods and Tables I and II). After overnight stimulation with LPS, the cells were washed thoroughly, treated with mitomycin C, and used as stimulators in triplicate cultures (3:1 responder:stimulator ratio) for C3H spleen cells (pooled from four stock mice). Cultures in the upper half of the table were incubated in the presence of pooled normal rat Ig (10 μg/ml), whereas cultures in the lower half of the table were incubated in the presence of 10 μg/ml anti-OX2 (3B6). Cells were harvested at 5 days and added to fresh cultures containing 5 × 10^6 C3H responder cells and 2 × 10^6 fresh C57BL/6 as in Table II.

† As for Tables I and II.

*, p < 0.05 compared with control in first row.

DC incubated with antisense ODNs for MD-1 induce “suppressor cells” for type 1 cytokines

DC incubated with ODN-1 show decreased expression of CD80/CD86, but expression of OX2 is preserved (Fig. 2). Since we reported that expression of OX2 by DC typifies a population of DC capable of inducing cells that can suppress type 1 cytokine production by T cells stimulated with conventional “stimulatory” DC (19), we asked whether cells generated in cultures of spleen cells stimulated with ODN-1-treated (OX2+) DC were able to suppress an MLR response from freshly stimulated responder T cells. Bone marrow-derived C57BL/6 DC were incubated in the presence of ODN-1 or ODN-3 as above, cultured overnight with LPS, and then used as stimulator cells for normal C3H spleen responder cells for 5 days (3:1 responder:stimulator ratio). Cells were harvested from these cultures or from control cultures receiving no DC and added to secondary cultures of fresh C3H spleen responder cells and fresh C57BL/6 (experimental) or BALB/c (control) bone marrow-derived DC. Control (secondary) cultures received only the responder cells and fresh DC. Proliferation, CTL, and cytokine production was assayed as before. Data for one of three such studies are shown in Table III.

It is clear from Table III that DC pretreated with ODN-1 and used as stimulator cells in culture do indeed induce a population with Ag-specific suppressive potential as assayed in a second MLR culture; note the failure to inhibit responses stimulated by BALB/c DC. By contrast, cells taken from primary cultures that used untreated DC, or DC treated with ODN-3, had no such suppressive potential.

Role of OX2 expression in suppression of MLR caused by ODN-1-treated DC

To test whether suppression generated by ODN-1-treated DC resulted from the persistent expression of OX2 by these DC, at the expense of expression of CD80 and CD86, we examined whether the addition of anti-OX2 mAb to ODN-1-altered DC prevented those cells from inducing the suppression illustrated in Table III. Data from one of three such studies are shown in Table IV. It is clear from this table that anti-OX2 mAb did indeed block the induction of suppression.

Increased C57BL/6 renal allograft survival following pv infusion of ODN-1-treated C57BL/6 DC

In a final assay, we investigated whether ODN-1-treated DC (with reduced expression of CD80/CD86, but persistent expression of OX2) would induce prolongation of renal allograft survival after pv immunization, consistent with the proposed role for OX2 in graft enhancement in vivo (15, 18). C57BL/6 bone marrow-derived DC were incubated with medium alone, ODN-1, or ODN-3 as before. Cells were further stimulated overnight with LPS, and 5 × 10^6 cells infused via the pv into groups of C3H/HeJ recipients, which subsequently received C57BL/6 renal allografts. The mice also received an infusion of control rat Ig, or 3B6 Ab (100 μg/mouse, i.e. at 2-day intervals for a total of five injections, beginning on the day of transplant). Survival was monitored daily. Data in Fig. 4 show cumulative data for a total of six mice per group.

As expected, pv infusion of bone marrow-derived DC led to increased renal allograft survival (○), an effect that was reversed by infusion of anti-OX2 mAb (●). Interestingly, infusion of ODN-1-treated DC (△), but not ODN-3-treated DC (□), led to a further

![Figure 4](http://www.jimmunol.org/)  
**FIGURE 4.** ODN-treated DC further increase renal allograft survival after pv immunization, an effect that is abolished by anti-OX2 mAb. See Materials and Methods for more details. Groups of five C3H mice received pv immunization with 5 × 10^6 C57BL/6 DC pretreated for 6 h with different ODNs (or with medium only; see Figs. 1 and 2), followed by C57BL/6 renal allografts. Thereafter, mice were given i.v. immunizations at 2-day intervals (total of five injections) with 100 μg anti-OX2 (3B6) or control normal rat Ig. A control group of transplanted mice received no pv immunization, again with/without infusion of anti-OX2 (● and ▲). The group shown as (△) showed increased survival relative to (○ and □), p < 0.05; all groups (△, ○, and □) showed increased survival relative to the unimmunized controls (▲, ▼), p < 0.05, Mann-Whitney U test.
enhancement in graft survival in vivo ($\rho < 0.05$, Mann-Whitney $U$ test). Increased survival in DC-treated mice, but not control mice (compare ▼ and ▼), was abolished in all cases by infusion of 3B6 mAb (▲, ■).

**Discussion**

The data in this paper show that MD-1 is not associated directly with CD80/CD86, DEC205, or OX2 on the membrane of functional DC (Fig. 3). However, inhibition of MD-1 synthesis achieved by incubating DC with the ODN-1 blocked MD-1 synthesis and then indirectly inhibited up-regulation of the costimulatory molecules CD80/CD86 in response to LPS (Fig. 2). These ODN-1-altered DC-stimulated Th2-type cytokine production instead of Th1 cytokines when cultured with allogeneic responder splenocytes (Table I), and proliferation and CTL generation was not seen. Instead, there was generation of cells that could suppress in a secondary test culture the allogeneic response of untreated C3H splenic T cells to unaltered allogeneic DC (Table II). Generation of these suppressor cells was dependent on persistent OX2 expression on the ODN-1-treated DC, because anti-OX2 mAb blocked the effect. The ability of ODN-1-treated DC to enhance allograft survival in vivo via an OX2-dependent pathway (Fig. 4) supports the view that OX2 on DC delivers an inhibitory signal, whereas MD-1, via indirect up-regulation of CD80/CD86 in response to other inflammatory stimuli (in the experimental case here, from LPS stimulation), delivers an opposing graft rejection stimulus to the recipient.

DC deprived of MD-1 by treatment with ODN-1, and thus of enhanced CD80/CD86 expression following LPS stimulation, had preserved levels of OX2 and proved particularly effective when injected via the pv system in prolonging allograft survival. These data are reminiscent of the synergistic increase in allograft survival we reported when infusion of OX2+ DC was combined with mAbs to CD80 and CD86 (18). In this study, ODN-1 is itself contributing to the decrease in functional expression of CD80/CD86 (see Figs. 1 and 2). Although we did not test the function of DC on which MD-1 had been removed by capping, it would be predicted that such cells would still stimulate the expected Th1 cytokine, proliferative, and CTL responses mediated by CD80/CD86, whose levels remain unperturbed (Fig. 3). To conduct such an experiment, the capped DC would need to be fixed, e.g., with glutaraldehyde, to prevent regeneration of surface MD-1 and CD14 that occurs within 12–18 h (R. M. Gorczynski, unpublished data). As this experiment has not yet been done, it is not possible to exclude a direct costimulatory role for MD-1 on T cells. In addition, it is important to note that we have not investigated other functional properties of these ODN-treated, LPS-stimulated, DC, including, for instance, production of IL-12, which has itself been implicated in the regulation of type 1 cytokine production (20).

In our experiments, we have examined surface marker expression and function of bulk populations of DC. It is known that OX2 is only expressed on a small proportion of DC in bulk bone marrow DC cultures (19). By double staining and flow cytometry, OX2+ cells are both CD80+ and CD80+ (R. M. Gorczynski, unpublished data). MD-1 expression on these cells has not yet been examined in detail. However, we favor the notion that MD-1+ DC do not express OX2, because otherwise one might have expected that MD-1-mediated activation signals that up-regulate CD80/CD86 indirectly (Figs. 1 and 2) would, directly or indirectly, affect OX2 expression. This was not seen (see Figs. 1 and 2).

MD-1 and MD-2, which have also now been cloned and characterized (34), appear to be members of a family of molecules. MD-1 was originally reported as a v-myb-regulated gene (30). MD-1 is a secreted molecule, but can be tethered to the cell surface when it is expressed in association with members of a family of molecules expressing an extracellular LRR motif (23). Among the latter are, on B cells, RP105, which transmits an activation signal to B cells after cell surface stabilization by MD-1 (21), and members of the Toll-like receptor family, involved in conferring intracellular activation signals following LPS activation, on macrophages/DC (21, 25, 30). These LRR molecules in general form a family of PRRs and are implicated in signaling for innate immunity following triggering by common conserved motifs on pathogens, i.e., “danger”. In turn, signaling via LRR-bearing molecules (in association with the MD family of molecules) leads to NF-κB activation and has been reported to be associated with regulation of expression of members of the CD80/CD86 family (25). Thus c-rel knockout mice show decreased B cell activation after cross-linking of RP105 (21). We speculate that infusion of cells into the pv represents a stimulus triggering PRRs, with concomitant increased expression of MD-1, which in turn favors increased expression of CD80/CD86. What a PRR might “see” on allogeneic stimulator cells that signals “danger” is unknown. Up to now, TCR interaction with MHC alloantigen was thought sufficient to lead to a Th1 cytokine/graft rejection response. Now there is evidence of other recognition events important in regulating such responses. The choice of Th1 vs Th2/3 cytokine production by T cells responding to Ag, which has been attributed to an inherent structure of particular MHC-binding peptides, may be determined by the recognition by cells of the innate immune system. Specific experiments to test this idea are in progress.

In summary, our data suggest that renal allograft rejection following donor-specific pv immunization is a net result of competing processes, the one (mediated by OX2) leading to immunosuppression, whereas the other, regulated by increased expression of MD-1, and thus in turn of CD80/CD86, leads to increased graft rejection. Accordingly, we propose that optimal graft survival will result from blocking the latter (e.g., as here, using ODNs to MD-1), and facilitation of the former (e.g., by using OX2Fc, or similar immunosuppressive regimes).

**References**


