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Evidence That an OX-2-Positive Cell Can Inhibit the Stimulation of Type 1 Cytokine Production by Bone Marrow-Derived B7-1 (and B7-2)-Positive Dendritic Cells

Laura Gorczynski, Z. Chen, J. Hu, Y. Kai, J. Lei, V. Ramakrishna, and R. M. Gorczynski

We reported that hepatic mononuclear, nonparenchymal cells (NPC) can inhibit the immune response seen when allogeneic C57BL/6 dendritic cells (DC) are incubated with C3H spleen responder cells. Cells derived from these cultures transfer increased survival of C57BL/6 renal allografts in C3H mice. We also found that increased expression of OX-2 on DC was associated with inhibition of cytokine production and renal allograft rejection. We explored whether inhibition by hepatic NPC was a function of OX-2 expression by these cells. Fresh C57BL/6 spleen-derived DC were cultured with C3H spleen responder cells and other putative coregulatory cells. The latter were derived from fresh C3H or C57BL/6 liver NPC, or from C3H or C57BL/6 mice treated for 10 days by i.v. infusion of human Flt3 ligand. Different populations of murine bone marrow-derived DC from cultures of bone marrow with IL-4 plus granulocyte-macrophage-CSF were also used as a source of putative regulator cells. Supernatants of all stimulated cultures were examined for functional expression of different cytokines (IL-2, IL-4, IFN-γ, and TGFβ). We found that fresh C57BL/6 splenic DC induced IL-2, not IL-4, production. Cells from the sources indicated inhibited IL-2 and IFN-γ production and promoted IL-4 and TGFβ production. Inhibition was associated with increased expression of OX-2 on these cells, as defined by semiquantitative PCR and FACS analysis. By size fractionation, cells expressing OX-2 were a subpopulation of NLDC145+ cells. Our data imply a role for cells expressing OX-2 in the regulation of induction of cytokine production by conventional allostimulatory DC. The Journal of Immunology, 1999, 162: 774–781.

Experimental and clinical studies suggest that techniques producing allo-specific delayed graft rejection generally result in a relative unresponsiveness in those CD4+ T cell subsets responsible for the production of so-called type 1 cytokines (IL-2 and IFN-γ) with selective sparing of type 2 cytokine-producing (IL-4, IL-10, and TGFβ) cells. A number of laboratories, including our own, have suggested that Ag-specific preimmunization via the portal vein (pv) is one protocol that produces increased graft survival and preferential type 2 cytokine production in animals (1–4). We have hypothesized that the mechanism(s) operating to induce tolerance after pv immunization reflect similar events to those occurring after oral immunization (5–7).

How such polarization in CD4+ cytokine-producing cells develops in vivo (8, 9) remains an enigma. Stimulation of T cells depends not only on the cytokine milieu in which Ag recognition occurs, but also on the nature of the APC and the level of expression of various costimulator molecules on those APC. Using a model in which we infused long-term culture-derived donor dendritic cells (DC) as tolerogen, we have also reported that persistent graft survival was dependent upon the existence of functional donor Ag in association with host DC (10). Furthermore, we found that stimulation of spleen responder cells with allogeneic DC in the presence of syngeneic (with the responder cell source) hepatic nonparenchymal mononuclear cell (NPC) preparation led to inhibition of type 1 cytokine production, development of a cell population able to inhibit further stimulation (to type 1 cytokine production) in vitro by fresh DC, and prolonged graft survival in vivo (11). These data were consistent with the notion that induction of suppression of type 1 cytokine production in vitro, and increased graft survival in vivo, is associated with the existence of a “facilitator” cell in the NPC preparation that somehow regulates the functional outcome of allostimulation.

More recent studies have attempted to explore whether expression of novel molecules on APC following pv immunization might be associated with the increased renal graft survival seen in this model. We reported that expression of OX-2, a previously described DC surface Ag (12–14), was increased in NLDC145+ cells taken from pv-immunized mice and that anti-OX-2 Ab abolished the increased graft survival following pv immunization (15). Interestingly, in a separate study, Borriello et al. also reported that OX-2 provided a costimulatory signal for activated murine T cells, but was unable to increase IL-2 production (16). Our data suggest that OX-2 in fact provides a costimulatory signal for preferential IL-4 (TGFβ) production (15). Taken with the observations using syngeneic NPC, we have thus asked whether the “facilitator” cell that alters the outcome of stimulation of spleen responder cells with allogeneic DC is itself an OX-2-expressing cell population. The experiments described below are derived from a series of studies designed to test this hypothesis. In addition, we have made use of a recently described growth factor, Flt3 ligand (Flt3L) (17–19), which is known to increase the proliferation of hematopoietic progenitor cells, to show that mice infused with human Flt3L contain increased numbers of OX-2+ cells in the liver NPC pool and that these latter cells alone, infused i.v. into naive recipients, can induce prolonged survival of renal allografts.
Materials and Methods

Mice

Male and female C3H/HEJ and B10.BR (H-2b/k), B10.D2 (H-2d/d), and C57Bl/6 (H-2d/d) 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.

Monoclonal Abs

The following mAbs, all obtained from PharMingen (San Diego, CA) unless stated otherwise, were used: anti-IL-4 (JES6-1A12; biotinylated, JE6-5H4); anti-IL-4 (11B11, American Type Culture Collection, Manassas, VA; biotinylated, BV6-24G2); anti-IFN-γ (R4-8A2, American Type Culture Collection; biotinylated XM1G2); anti-IL-10 (JES5-2A5; biotinylated SXC-1); and phycoerythrin anti-B7-1/B7-2 (Cedarlane Labs, Hornby, Ontario, Canada).

Rat anti-mouse OX-2 mAbs were purchased from Immuno-Precise Antibodies (Victoria, British Columbia, Canada) following immunization of rats with a crude membrane extract of LPS-stimulated murine DC, followed by fusion with a nonsecreting rat myeloma parent cell line (YBE2/3H1.P2.G11.16Ag.20). Hybridoma supernatants were screened in ELISA using plates precoated with a 40- to 45-kDa preparation of DC extracts run on Western gels (13). Positive clones were then expanded with the aid of FACS analysis of Chinese hamster ovary (CHO) cells transduced with a cDNA clone encoding full-length murine OX-2 (14). FITC-conjugated F(ab)2 rabbit anti-rat IgG (noncross-reactive with mouse IgG) from Serotec (Mississauga, Canada) was used as secondary Ab. The mAb selected for further analysis (M3B5) was grown in bulk in a CellMax system (Cellco, Germantown, MD). A crude preparation of rat Ig (30% saturated ammonium sulfate preparation) was used as a control Ig.

In tissue culture assays, where anti-cytokine mAbs were used to confirm the specificity of the assay used, 10 μg/ml of the relevant mAbs was found to neutralize up to 5.0 ng/ml of the cytokine tested.

NLDCl45 (anti-mouse DC) was also obtained from Serotec. Recombinant mouse IL-4 was a kind gift from Dr. D. L. Yang (The Toronto Hospital); mouse recombinant granulocyte-macrophage-CSF was purchased from PharMingen. Recombinant human Flt3L (derived from CHO cells) was a kind gift from Dr. A. B. Troutt (Immunex, Seattle, WA).

Renal transplantation

Renal transplantation was performed essentially as described elsewhere (20). Animals were anesthetized with a combination of halothane and nitrous oxide inhalation using novocain for postoperative analgesia. Orthotopic transplantation was performed using routine procedures. In brief, donor animals received 200 U heparin, and kidneys were flushed with 2 ml of ice-cold heparinized physiological saline solution before removal and transplantation into recipient animals with left nephrectomy. The graft renal artery was anastomosed to the recipient’s aorta, and the renal artery was anastomosed to the recipient’s inferior vena cava. The renal vein was anastomosed to the recipient’s vena cava. The ureter was sutured to the recipient’s bladder using a small double-armed polyethylene 5-0 patch. All recipients received i.m. injection of cefotetan (30 mg/Kg) on the day of transplantation and for 2 successive days. The remaining host kidney was removed 2 days after transplantation, unless otherwise indicated. Treatment of recipients with pv immunization, by mAbs, or by oral immunization was as described in individual studies.

Portal vein and oral immunization

Portal vein and oral immunization was performed as described earlier (9, 10). All animals were anesthetized with nembutal. A midline abdominal incision was made and the viscera exposed. Cells were injected in 0.1 ml volume of 100 μl saline and transplanted into recipient animals with left nephrectomy. Treatment of recipients with pv immunization, by mAbs, or by oral immunization was as described in individual studies.

Cytotoxicity and cytokine assays

In cultures used to assess induction of cytotoxicity or cytokine production, responder cells were stimulated with irradiated (2000 rad) stimulator cells in 96-well plates and harvested after 48 h for cytokine assays (see below). No reproducible differences in cytokine levels have been detected from cultures assayed between 36 and 54 h of stimulation. In some experiments, the cultures received 1 μCi/well (at 72 h) of [3H]TdR, and proliferation was assessed by harvesting cells 14 h later and counting in a well-type beta-counter.

Where cytotoxicity was measured, cells were harvested and pooled from equivalent cultures at 5 days, counted, and recultured at different E:T ratios with 3Cr EL4 (H-2b/d) or P815 (H-2b/d) tumor target cells. Supernatants were sampled at 4 h for assessment of specific cytotoxicity.

IL-2 and IL-4 activity were assessed by bioassay using the IL-2/IL-4-dependent cell lines, CTLL-2 and CT4.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 to block potential stimulation of CTLL-2 with IL-2. IL-4 assays were set up in the presence of S4B6 to block IL-2-mediated stimulation. Both the IL-2 and IL-4 assays reproducibly detected 50 pg of recombinant lymphokine added to cultures.

In addition, IL-2, IL-4, IFN-γ, and IL-10 were assayed using ELISA assays. For IFN-γ, the assay used flat-bottomed Nunc plates (Life Technologies, Grand Island, NY) coated with 100 ng/ml R4-6A2. Varying dilutions of supernatant were bound in triplicate at 4°C, washed three times, and biotinylated anti-IFN-γ (XM1G2) added. After washing, plates were incubated with streptavidin-horseradish peroxidase (Cedarlane Labs), developed with appropriate substrate, and OD405 determined using a ELISA plate reader. IFN-γ was standardized from Pharmingen. IL-10 was similarly assayed by ELISA, using JES5-2A5 as a capture Ab and biotinylated SXC-1 as developing Ab. IL-R10 for standardization was from PepTec (Rocky Hill, NJ). Each assay detected 0.1 ng/ml cytokine.

ELISA assays for IL-2 and IL-4 used JES6-1A12 and 11B11 as capture Abs, with JAS6-5H4 or BV6-24G2 as developing Abs. Sensitivity of detection was 20 pg/ml for each cytokine. Where checked, the correlation between bioassay and ELISA for IL-2 or IL-4 was excellent (r > 0.90). In all studies reported below, data are shown from ELISA assays only. Where cytokine data are pooled from several studies (e.g., Figs. 6, 8, 9), absolute values of cytokine production were obtained as above using commercial recombinant cytokines to standardize the assays. In our hands, supernatants from C3H anti-C57Bl/6 cultures, under the conditions described, reproducibly contain 950 ± 200 and 8 ± 25 pg/ml IL-2 and IL-4, respectively.

Preparation of RNA

Different sources of tissue from renal-grafted female mice receiving DC and kidney allografts from male mice were harvested for RNA extraction as described elsewhere (9). The OD260/280 of each sample was measured, and reverse transcription was performed using oligo (dT) primers (27-7858; Pharmacia, Piscataway, NJ). The CDNA was diluted to a total volume of 100 μl with water and frozen at −70°C until use in PCR reactions with primers for murine GAPDH, B7-1, B7-2, or OX-2. The sense and anti-sense primers were synthesized by the Biotechnology Service Centre (Hospital for Sick Children, Toronto, Canada) using published sequences. 5′ primers were [32P] end-labeled for PCR and had comparable levels of sp. act. after purification by ethanol precipitation. CDNA (5 μl) was amplified for 35 cycles and samples were analyzed in 12.5% polyacrylamide gels followed by overnight (18 h) exposure for autoradiography. In control studies, using H-Y primer sets, this technique reliably detects H-Y mRNA from extracts of female spleen cells to which male cells are added at a concentration of 1:105 (10). Quantitative comparison of expression of different PCR products used densitometric scanning of the autoradiograms: GAPDH sense, 5′-TGATGACATCAAGAAGGTTGGAAG-3′; GAPDH anti-sense, 5′-CTCTGTTGAGCCATGTAGGCCAT-3′; B7-1 sense, 5′-CCTGCGTGTCACCTCTCC-3′; B7-1 anti-sense, 5′-CGGAAGACAGAAGCAGTAAC-3′; B7-2 sense, 5′-TCTCAGATGCTGTTTCCG-3′; B7-2 anti-sense, 5′-GGTTTCACTGAAATGGTGGCAG-3′; OX-2 sense, 5′-GGTGAAGTGTTGAGCCAGGA-3′; OX-2 anti-sense, 5′-ATAGAGAGTAAGCAAGCTG-3′.
B10.BR (H2 k/k ) bone marrow-derived DC in the presence/absence of cells alone (group 1), or with 2 × 10^5 B10.BR DC (group 2). Further groups (3–5 and 6–8, respectively) contained C57BL/6 responder cells and 2 × 10^5 NPC from either C57BL/6, B10.D2, or B10.BR, respectively (3–5), or these same NPC and B10.BR DC (6–8). Data show mean proliferation and cytokine production from triplicate cultures in three separate studies. B. Data show proliferation and cytokine production from cultures of 5 × 10^5 C57BL/6 responder spleen cells stimulated in triplicate with 2 × 10^5 B10.BR DC alone, or with the addition of 2 × 10^5 cells harvested from the cultures shown in the A. Again, data represent arithmetic means of three separate experiments. * p < 0.05 compared with control cultures (far left in each panel).

**Statistical analysis**

In studies with multiple groups, ANOVA was performed to compare significance. In some cases (as defined in individual circumstances), pairwise comparison between groups was also subsequently performed.

**Results**

Ag stimulation, in the presence of hepatic NPC, induces development of a cell population capable of inhibiting proliferation and IL-2 production on adoptive transfer

In a previous manuscript (11), we reported that C3H spleen cells, stimulated in the presence of syngeneic NPC and allogeneic (C57BL/6) DC, produced a cell population capable to inhibit generation of IL-2 from fresh spleen cells stimulated with C57BL/6 DC and capable of inhibiting C57BL/6 renal allograft rejection in vivo. To ask whether this function of NPC was MHC restricted or not, we performed the following study.

C57BL/6 (H2^b) spleen cells were stimulated in vitro with B10.BR (H2^k) bone marrow-derived DC in the presence/absence of the following NPC: C57BL/6, B10.BR, and B10.D2 (H2^k). In addition, control cultures were incubated with the NPC only. Proliferation and IL-2/IL-4 production was measured in one aliquot of these primary cultures. In addition, at 5 days cells were harvested from another set of the primary cultures, washed, and 2 × 10^5 cells added to cultures containing 5 × 10^6 fresh C57BL/6 spleen cells and B10.BR DC. Proliferation and cytokine production was measured in these latter cultures in standard fashion. Data pooled from three equivalent studies are shown in Fig. 1, A and B.

There are a number of points of interest. As previously documented, the addition of NPC syngeneic with spleen responder cells (C57BL/6 in this case) to cells stimulated with allogeneic (B10.BR) DC led to decreased proliferation and IL-2 production from those responder cells compared with cells stimulated by DC alone (compare groups 6 and 2 of Fig. 1A). In contrast, IL-4 production was enhanced. NPC alone, whether syngeneic or allogeneic to the responder cells, produced no obvious effect (groups 3–5, Fig. 1A). Furthermore, cells from primary cultures receiving the DC + NPC mixture were able to inhibit proliferation and IL-2 production (while promoting IL-4 production) from fresh spleen cells stimulated in secondary cultures with the same (B10.BR) DC (see Fig. 1B). However, the data in Fig. 1 make another important point. The same inhibition of proliferation/IL-2 production in primary cultures was seen using either B10.BR NPC (MHC matched with the DC stimulus; group 8, Fig. 1A) or with third-party B10.D2 NPC (MHC mismatched with both spleen responder cells and allogeneic stimulator DC; group 7, Fig. 1A). Again no obvious effect was seen in cultures stimulated with B10.BR or B10.D2 NPC alone (groups 4 and 5, Fig. 1A). Finally, cells taken from primary cultures stimulated with DC and NPC from either B10.BR or B10.D2 could also inhibit proliferation/IL-2 production from secondary C57BL/6 spleen cell cultures stimulated with B10.BR DC; again, cells taken from primary cultures with NPC alone produced no such inhibition (Fig. 1B). Thus, the inhibition of proliferation/IL-2 production and enhancement of IL-4 production seen in primary cultures, as well as the induction of suppression measured in secondary cultures, all induced by NPC, are not MHC restricted.

Specificity of inhibition/suppression induced by hepatic NPC

One interpretation of the data shown in Fig. 1 and elsewhere is that NPC deliver a signal to DC-stimulated cells that is distinct from the Ag-specific signal provided by the DC themselves (and is MHC nonrestricted). This signal modulates the Ag-specific signal provided by the DC. To assess the Ag-specificity of the immunoregulation described in Fig. 1, the following experiment was performed.

C57BL/6 spleen responder cells were stimulated with B10.D2 or B10.BR bone marrow-derived DC in the presence/absence of NPC from B10.BR or B10.D2 mice. Proliferation and cytokine production was measured in aliquots of these cultures as before. In addition, further aliquots of cells harvested from these primary cultures were added to cultures of fresh C57BL/6 spleen cells stimulated with B10.BR (Fig. 2B) or B10.D2 (Fig. 2C) DC. Again, proliferation and cytokine production was measured. Data pooled from three such studies are shown in Fig. 2.

Data from the primary cultures (Fig. 2A) recapitulates the observations made in Fig. 1 and show that NPC inhibit proliferation and IL-2 production from DC-stimulated responder cells in an Ag- and MHC-unrestricted fashion. However, the data in Fig. 2, B and C clearly show that adoptive transfer of inhibition using cells from these primary cultures occurs in an Ag-restricted fashion, dictated by the Ag-specificity of the DC used in the primary cultures, not of the NPC used for induction of suppression. Thus, these auxiliary cells in the NPC population have a functional property of being “facilitator cells for induction of suppression.” Note that in other studies (data not shown) where the final assay system involved measuring cytotoxicity to allogeneic target cells, a similar inhibition of lysis (rather than cytokine production) was seen using cells harvested from primary cultures stimulated with DC and hepatic NPC (see Ref. 11).

Hepatic cell preparations from Flt3L-treated mice are a potent source of DC and “facilitator” cells

We have reported at length that pv infusion of alloantigen, or i.v. infusion of liver-derived allogeneic mononuclear cells induces operational unresponsiveness in recipient animals (10, 20, 22, 23). The total hepatic mononuclear cell yield from normal mice is of...
the order of $5 \times 10^6$ cells/mouse. To increase the yield, and explore the possibility that the liver itself might be a source both of allostimulatory DC and “facilitator” cells, we exposed two C57BL/6 mice for 10 days to daily i.v. infusions of $10^3$ mg/mouse human CHO-derived Flt3L, a known growth factor for DC (18). Liver tissue was harvested and pooled from these donors, and mononuclear cells were prepared as described in Materials and Methods (mean $1.3 \times 10^8$ cells/donor). These cells were further subjected to subfractionation by size using unit gravity sedimentation techniques (24). A typical size profile for recovered cells is shown in Fig. 3 (one of three studies).

In these same studies, cells isolated from the various fractions shown in Fig. 3 were tested as follows. Firstly, cells were stained with FITC-labeled mAbs to B7-1, B7-2, NLDC145, and rat anti-mouse OX-2 (M3B5) with FITC anti-rat IgG as secondary Ab. In addition, mRNA extracted from the different cell samples were assayed by PCR for expression of GAPDH, B7-1, B7-2, and OX-2. Data are shown in Figs. 3 (pooled from three separate studies) and 4 (representative PCR data from one experiment). Further aliquots of the cells were used to stimulate fresh C3H spleen responder cells in culture. Proliferation and cytokine assays were performed as before (Fig. 1), and, in addition, cells were taken from these primary cultures and added to fresh secondary cultures of C3H spleen responder cells and C57BL/6 bone marrow-derived DC. Again, proliferation and cytokine production was assayed from these secondary cultures. Data pooled from three studies of this type are shown in Fig. 5, A and B. Finally, cells from the various fractions were infused i.v. into two C3H mice per group, which also received C57BL/6 renal allografts as Ag challenge. Spleen cells were harvested from these individual mice 10 days after transplantation and restimulated in culture with C57BL/6 or B10.D2 DC, again with cytokines measured at 40 h (see Fig. 6).

FIGURE 2. Specificity of inhibition of proliferation or cytokine production by hepatic NPC (see text and Fig. 1 for more details). A, $5 \times 10^6$ C57BL/6 spleen cells were stimulated in triplicate for 3 days with $2 \times 10^6$ B10.BR or B10.D2 DC with/without $2 \times 10^5$ NPC derived from B10.D2 or B10.BR mice. Data shown are arithmetic means of three repeat studies. B and C, fresh C57BL/6 responder spleen cells were cultured in triplicate with either B10.BR DC (B) or B10.D2 DC (C) with/without $2 \times 10^5$ additional cells from the primary cultures (groups 1–6 in A). Again, data represent arithmetic means of proliferation/cytokine production from three studies. *, $p < 0.05$ compared with control cultures (far left in each panel).
Data in Fig. 3 show that distinct subpopulations of slow-sedimenting cells express OX-2 in the cells harvested from Flt3L-treated mice when compared with cells expressing B7-1 and/or B7-2. In general, expression of OX-2 and B7-2 occurred in equivalent subpopulations. Faster-sedimenting cells (Fx3 and Fx4 in Fig. 3), while staining for NLDC145, were positive by fluorescence mainly for B7-1, not B7-2 or OX-2. Similar conclusions were reached both by FACS analysis of cell populations (Fig. 3) and by PCR analysis of mRNA (Fig. 4).

When the functional capacity of these different cell populations was investigated (Figs. 5 and 6), it was found that optimal direct stimulation (or proliferation and IL-2 production) was seen from B7-1-expressing cells (Fx3 and Fx4 in Fig. 5A), while only OX-2-expressing cells (Fx1 and Fx2 in Figs. 3 and 4) were capable of producing the inhibitory effects defined earlier (Figs. 1 and 2) in the two-stage culture system (Fig. 5B). These same cells (Fx1 and Fx2) were in turn able, after i.v. infusion, to polarize cells from mice given renal allografts to produce predominantly IL-4, IL-10, and TGFβ production on restimulation in vitro (Fig. 6). These data are consistent with the notion that after Flt3L treatment of mice expansion of a population of immunostimulatory DC occurs within the liver, which also contains another distinct population of “facilitator” cells that promote immunoregulation.

Evidence that cell populations with “facilitator” activity from the liver of Flt3L-treated mice prolong graft survival in vivo

Because we have reported elsewhere that there is a good correlation between treatments (such as pv immunization) that decrease IL-2 production and increase IL-4 production from restimulated cells and prolongation of graft survival (4, 10), and that increased expression of OX-2 is also independently associated with increased graft survival after pv immunization (15), we next asked whether cells isolated from Flt3L-treated mice, which induced inhibitory function in vitro (see Figs. 1, 2 and 5) and expressed increased amounts of OX-2 (Figs. 3 and 4), were themselves capable of promoting increased graft survival in vivo.

Groups of two C57BL/6 mice received i.v. infusions of 10 μg/mouse Flt3L for 10 days as before. Cells were isolated from the liver by enzyme digestion and fractionated by unit gravity sedimentation. Four pools of cells were recovered, and an aliquot stained as before in FACS with anti-OX-2. Groups of two C3H mice received 10 × 10^6 cells i.v. from the four separate pools. A control group received saline injections only. Over the next 48 h, all mice received C57BL/6 renal transplants. All mice received cyclosporin (CsA) (10 mg/Kg) on the day of renal transplantation. The data in Fig. 7 are pooled from three studies of this type (representing six mice/group) and show the animal survival in these five different groups.

It is quite clear from Fig. 7 that only hepatic cells expressing OX-2 (Fx1 and Fx2; see Figs. 3 and 4) were capable of promoting increased graft survival after i.v. infusion. Comparison of these data with those in Fig. 5 confirm that these cell populations were also those identified, using a two-stage culture assay system, as cells with functional “facilitator” activity (see also Figs. 1 and 2). Note that though our conclusions are restricted by the use of only six mice/group in this study, there was no significant difference in survival between groups receiving NPC-Fx1 or NPC-Fx2 in this experiment, in keeping with relatively equivalent levels of OX-2 expression in these fractions (Fig. 3).

Anti-OX-2 mAb in vitro reverses regulation induced by hepatic NPC

In a final study, we asked whether anti-OX-2 mAb M3B5, added to cultures of C3H spleen responder cells, allogenic (C57BL/6) DC and NPC from C57BL/6 mice, could prevent the inhibition of IL-2 production in primary cultures, and the development of cells able to inhibit such cytokine responses from freshly stimulated responder cells in secondary cultures (see Figs. 1, 2, and 5). Data in Figs. 8 and 9 are pooled from three studies of this type. Primary cultures were of two types, containing C3H responder spleen cells and C57BL/6 DC alone (Fig. 8), or the same mixture with added C57BL/6 NPC (Fig. 9). Subsets of these cultures contained in addition either 5 μg/ml of anti-B7-1, anti-B7-2, or anti-OX-2. Supernatants from responder cells stimulated in the presence of DC only were assayed after 60 h for cytokine production (Fig. 8). For the primary cultures incubated with both DC and NPC, supernatants were harvested at 60 h and tested for cytokine production (Fig. 9A). In addition, cells were harvested after 5 days, washed,
and added to secondary cultures of fresh C3H responder cells with fresh C57BL/6 DC. No mAbs were added at this second culture stage. Data for cytokine production of these secondary cultures are shown in Fig. 9B.

The addition of anti-B7-1 or anti-B7-2 to DC-stimulated spleen cultures led to inhibition of cytokine production (Fig. 8), while in contrast anti-OX-2 mAb led an increase in IL-2 production in these primary cultures (Fig. 8). We have reported similar findings elsewhere (45). Interestingly, anti-OX-2 abolished the inhibition of cytokine production caused by NPC in these primary cultures (Fig. 9A; see also Figs. 1, 2, and 5). In addition, anti-OX-2 prevented the functional development of a cell population capable of transferring inhibition of cytokine production to freshly stimulated spleen cells (Fig. 9B).

Discussion

There is considerable theoretical as well as practical interest in understanding the mechanism(s) by which a state of Ag-specific tolerance can be induced in lymphoid populations. Limits to the effective induction of tolerance represent a major challenge to more successful allo- (and xeno-) transplantation, to name but one example (25). We and others have invested significant efforts into exploring how pre- (or peri-) transplant donor-specific immunization might produce such a state (1–3, 5, 26–28). There is good evidence that pv immunization somehow leads to tolerance induction, and this immunoregulation can apparently be monitored by following changes in cytokine production from host cells, with decreased production of IL-2, IL-12, and IFN-γ, and increased IL-4, IL-10, IL-13, and TGF-β (5, 11). Which, if any, of these cytokine changes is directly and causally implicated nevertheless remains obscure.

Further analysis of the cell population able to induce tolerance after pv immunization led to the somewhat paradoxical observation that donor DC represented an excellent tolerizing population (10). Because Ag-pulsed DC are conventionally thought of as representing an optimal immunizing regime, we were interested in the mechanism(s) activated following DC pv immunization that led to tolerance (29). It is already clear that DC themselves represent an extremely heterogeneous population, in terms of origin, cell surface phenotype, turnover in vivo, and possibly function (30, 31). In the mouse lymph node, at least three discrete populations were identified, one of which comprised small CD8α−CD11c145+ cells, likely of lymphoid origin, with an immature phenotype, and whose numbers were profoundly increased (100×) following Flt3L treatment in vivo (30) (administration of the latter has been reported to lead to proliferation of DC and other cells of hematopoietic origin (32)). These cells resembled the interdigitating DC found in the T cell areas of the splenic white pulp (31) and have been implicated in regulation of immunity induced by other (myeloid-derived) DC (30, 33, 34).

A variety of other studies have indicated that the induction of immunity (vs tolerance) following Ag presentation was intrinsically dependent upon the coexistence of other signaling ligands at the surface of DC (interacting with appropriate counterligands on the surface of other cells, e.g., stimulated T cells) (35–37). We speculated that infusion of DC via the pv induced tolerance by coopting another cell population, distinguishable by expression of unique cell surface ligands, whose biological function was to facilitate induction of tolerance, not immunity, when Ag was presented in association with otherwise immunogenic DC; some preliminary evidence supporting this hypothesis was recently reported (11). We have elected to refer to this as a facilitator cell. Moreover, because pv immunization has been shown to be associated with increased expression of a novel molecule, OX-2, previously reported to be expressed on DC (12, 14, 15), we wondered whether increased expression of a novel molecule, OX-2, previously reported to be expressed on DC (12, 14, 15), we wondered whether this molecule would in fact serve as a “marker” for the hypothetical facilitator cell described. Experiments reported above are consistent with such a hypothesis.

We have shown that within the hepatic NPC population there is a subset of cells able to inhibit stimulation by allogeneic DC in a non-MHC restricted fashion (see Figs. 1 and 2) and able to induce the development of an Ag-specific immunoregulatory cell population in vitro (see Figs. 1 and 2). The non-MHC-restricted nature of this “facilitator” cell interaction leads us to believe that it functions by providing an accessory signal (a regulatory, not a costimulatory, signal) to the DC that stimulates T cells in the allogeneic MLR we describe in a fashion analogous to the original description of costimulatory interactions (38). As a result, the stimulated lymphocytes alter their cytokine production profile (with decreased IL-2 production and proliferation), and become able to regulate the immune response seen from freshly stimulated lymphocytes (see Figs. 1B and 2B). Most interestingly, following expansion of DC in vivo by Flt3L treatment, we have been able to show that in fact the liver itself contains both an immunostimulating population (large cells by velocity sedimentation analysis) and this putative “facilitator” cell population (see Figs. 3–7). Furthermore, the latter biological activity resides within a slow-sedimenting (small size)
expressing cells be referred to as “facilitator” cells (for tolerance molecule seems to give cells the capability to induce immunoregulatory signals delivered following OX-2 expression. In a final attempt to explore the role for OX-2 expression itself in this regulatory function, we stimulated fresh spleen cells with DC alone or in the presence of anti-B7-1, anti-B7-2, or anti-OX-2. Note that other studies (data not shown) have confirmed that even the bone marrow-derived DC used contains small numbers of OX-2 cells (R.M.G., unpublished observations). Unlike anti-B7-1 and anti-B7-2, which decreased cytokine production, a result in keeping with the hypothesized role for these as costimulator molecules (39–41), anti-OX-2 produced a small but significant (1.7- to 2.5-fold in three studies) increase in IL-2 production in this system (Fig. 8). Most important, however, inclusion of anti-OX-2 mAb in a system where exogenous “facilitator” cells were added (from NPC) blocked completely the induction of inhibition normally seen in such cultures (Figs. 1 and 2; compare with Fig. 9B). These data are consistent with the concept that OX-2 delivers a regulatory, not a costimulatory, signal in this situation.

How do our data fit within the evolving framework of understanding in the heterogeneity of DC? As noted above, there has been speculation that a separate population of CD8α NLDC145 DC of lymphoid origin, which proliferates in response to Flt3L, might be responsible for immunoregulation. Other data have implicated IL-10 as a cytokine that might modify development/maintenance of DC into a population expressing increased amounts of B7-2 and capable of inducing tolerance (42). The role of regulation of expression of OX-2 as a controlling feature in this regard is unexplored (34). Our data is the first, to our knowledge, that implicates another molecule, OX-2, or its natural ligand, in the delivery of a tolerizing signal, perhaps in association with alterations in expression of B7-2, Fas, etc. It is intriguing that while there is clearly a key role for intrathymic DC in the regulation of self-tolerance (29), natural expression of OX-2 was initially first described on thymic DC (as well as within the brain) (12); there is as yet no evidence to suggest that this represents a functionally relevant expression for OX-2 in this location. However, other independent data have also implied an immunoregulatory role for OX-2 expression, again as assayed by altered cytokine production in vitro from cells stimulated in the presence/absence of expressed OX-2 (16).

We have also reported that following pv immunization there is a measurable expansion in numbers of populations of γδ TCR+ cells capable of adoptive transfer of increased graft survival to naïve recipients (43, 44). Little is known concerning the nature of the Ag recognized by these cells and why, as a population, their numbers are preferentially increased following pv immunization. We speculate that this may be explainable ultimately in terms of a differential susceptibility of γδ TCR+ vs βα TCR+ cells to immunoregulatory signals delivered following OX-2 expression.

In conclusion, we report for the first time that functional heterogeneity in the DC pool may be understandable in terms of differential expression of OX-2 on the cell surface. Expression of this molecule seems to give cells the capability to induce immunoregulation, increased renal graft survival (and altered cytokine production both in vivo and in vitro). We have suggested that such OX-2-expressing cells be referred to as “facilitator” cells (for tolerance induction). The mechanism by which they produce this effect, and the potential involvement of other signaling molecules at the cell surface (including Fas and perhaps the ligand for OX-2), remains to be explored.

References


