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Receptor Engagement on Cells Expressing a Ligand for the Tolerance-Inducing Molecule OX2 Induces an Immunoregulatory Population That Inhibits Alloreactivity In Vitro and In Vivo[1]

Reginald M. Gorczynski,[2]*† Kai Yu,* and David Clark*‡

Increased survival of C57BL/6 renal allografts following portal vein donor-specific pretransplant immunization of C3H mice is associated with increased expression of the molecule OX2 seen on host dendritic cells, along with a marked polarization in cytokine production from lymphocytes harvested from the transplanted animals, with preferential production of IL-4, IL-10, and TGF-β on donor-specific restimulation in vitro, and decreased production of IL-2, IFN-γ, and TNF-α compared with non-portal vein-immunized control transplanted mice. The increased renal allograft survival and the altered cytokine production are abolished by infusion of anti-mouse OX2 mAb (3B6). Infusion of a soluble OX2:Fc immunoadhesin can itself produce significant prolongation of xeno- and allografts in mice. We have used FITC-conjugated OX2:Fc to characterize cells expressing a ligand (OX2L) for OX2, and provide evidence that subpopulations of LPS-stimulated splenic macrophages, Con A-activated splenic T cells, and the majority (>80%) of $\gamma\delta$TCR$^+$ T cells express this ligand. We show below that F4/80$^+$, OX2L$^+$ splenic macrophages, admixed with OX2:Fc, represent a potent immunosuppressive population capable of causing more profound inhibition of alloreactivity in vitro or in vivo than that seen using either OX2:Fc or OX2$^+$ (or OX2L$^+$) cells alone. Immunoregulation by this OX2L$^+$ population occurs in an MHC-restricted fashion. ***The Journal of Immunology*****, 2000, 165: 4854–4860.**

Renal and skin allograft survival is increased following donor-specific portal vein (pv)[3] immunization (1–3), and is in turn associated with increased expression of a number of distinct mRNAs (4), one of which encodes OX2, a molecule expressed on the surface of dendritic cells (DC) (5). We showed subsequently that infusion of anti-OX2 mAbs from the time of transplantation blocks the protective effect of pv immunization in mice receiving renal allografts (4) and rats receiving small intestinal transplantation (6), and the polarization to type 2 cytokine production seen in these models (4, 6). A soluble immunoadhesin, in which the extracellular domain of OX2 was linked to a murine IgG2aFc region, inhibited T cell allostimulation and type 1 cytokine production (IL-2, IFN-γ) in vitro and in vivo (1). Because the intracellular domain of OX2 lacks signaling motifs, or any docking sites for adapter molecules that might engage an intracellular signaling cascade, we have suggested that these and other data (7) are consistent with the idea that engagement of the ligand of OX2 (OX2L) by OX2 may deliver key immunoregulatory signals.

T cells are activated after concomitant engagement of TCRs with Ag presented on APC in association with MHC molecules and the delivery of costimulatory signals resulting from the interaction of several ligand:coreceptor complexes (8–11). Major positive costimulatory interactions include the following: CD40L with CD40, and CD28 with CD80/CD86; CTLA4 interactions with CD80/CD86 may deliver a negative signal (12–17). Although positive costimulatory signals are clearly important in T cell triggering, blocking this costimulation alone, and/or facilitating signaling via CTLA4 has not reproducibly induced tolerance. This may reflect the need for other molecules (such as OX2) in active immunoregulation (4). In recent studies, we reported that DC expressing OX2 triggered an immunoregulatory function leading to increased allograft survival. Moreover, these cells were physically distinguishable from those DC with optimal allostimulatory capacity (7).

Early attempts to characterize a ligand for OX2 by Preston et al. (18) were performed by constructing a soluble chimeric protein with the extracellular domains of OX2 engineered onto domains 3 + 4 of rat CD4 Ag. The reagent was then complexed to fluorescent covaspheres to improve its ability to stain cells expressing OX2 ligand. These OX2 covaspheres were reported to bind to macrophages, but not other cell types. The specificity of the interaction was documented by inhibition studies using Fab of the OX2 mAb. Using site-directed mutagenesis, this group further reported results suggesting that the ligand-binding domain of OX2 was in the NH_2-terminal domain of the extracellular region of OX2.

Using FITC-labeled OX2:Fc, we report below that a ligand for OX2 does indeed exist on a subpopulation of LPS-stimulated splenic macrophages, but not fresh splenic DC. Moreover, subpopulations of Con A-activated $\alpha\beta$TCR$^+$ cells, and most $\gamma\delta$TCR$^+$ cells, also bind FITC-OX2:Fc. Admixture of OX2:Fc with the OX2L$^+$ splenic macrophage pool, followed either by in vivo infusion into renal transplant recipients, or by addition in culture to freshly allostimulated spleen responder cells produces profound inhibition of allorecognition, greater than that seen using either OX2:Fc or OX2$^+$ (OX2L$^+$) cells alone. Cells producing this inhibition copurify with small cells expressing the F4/80 surface

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[3] Abbreviations used in this paper: pv, portal vein; DC, dendritic cell; PLN, peripheral lymph node; PP, Peyer's patch.

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marker, and inhibition occurs in an MHC-restricted fashion. We conclude that optimal immunoregulation of transplant rejection in vivo may depend in part upon signals delivered by receptor cross-linking of an activated $OX2L^+$ subpopulation of $F4/80^+$ splenic cells.

Materials and Methods

Mice

Male C3H/HeJ, BALB/c, C57BL/6 and B10.Sgn, B10.BR, and B10.D2 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 were obtained from PharMingen (San Diego, CA), unless stated otherwise: anti-IL-2 (S4B6; American Type Culture Collection, Manassas, VA; biotinylated); anti-IL-4 (11B11; American Type Culture Collection; biotinylated); anti-IFN-γ (R4-6A2; American Type Culture Collection; biotinylated XMG1.2); anti-IL-10 (JES5-2A5; biotinylated, SXC-1). PE anti-CD80, PE anti-F4/80, FITC anti-αβTCR, FITC anti-γδTCR, L3T4 (anti-mouse CD4), anti-Thy-1.2, and anti-Ly-2.2 were obtained from Cedarlane Labs (Hornby, Ontario, Canada). The hybridoma-producing DEC205 (anti-mouse DC) was a kind gift from Dr. R. Steinman (Rockefeller University), and was directly labeled with FITC. $F(ab')_2$ rabbit anti-rat IgG PE conjugate (noncross-reactive with mouse IgG) was obtained from Serotec (Mississauga, Ontario, Canada). PE rat anti-mouse OX2 (3B6) was obtained from BioSpark (Mississauga, Ontario, Canada) (19).

Streptavidin HRP and mouse rGM-CSF were purchased from PharMingen.

Preparation of cells

Single cell spleen and peripheral lymph node (PLN) suspensions were prepared aseptically from individual mice in each experiment. After centrifugation, cells were resuspended in α-MEM supplemented with 2-ME and 10% FCS (αF10). Peyer's patch (PP) cells were used as a source of lymphocytes from which populations enriched for $\gamma\delta TCR^+$ cells were obtained by adherence for 90 min at 37°C to anti-γδTCR Ab-coated plates (20).

Fresh splenic DC were obtained as the nonadherent component of overnight culture of plastic adherent spleen cells, while splenic macrophages represented the persistently adherent pool (21). In addition, bone marrow-derived DC were prepared in vitro, as described elsewhere (22). Bone marrow cells were pooled from 10 donors, treated with the mixture of Abs (L3T4, anti-Thy-1.2, anti-Ly-2.2) and rabbit complement, and cultured in 10 ml αF10 in tissue culture flasks, at a concentration of 2×10^6/ml with 500 U/ml murine rGM-CSF (PharMingen). Fresh GM-CSF was added at 36-h intervals. Cells were separated over lymphopaque on days 3.5 and 7 of culture, again reculturing in αF10 with rGM-CSF. An aliquot of the sample stained at 10 days with FITC-conjugated DEC205 mAb showed a mean staining in the order of 91% ± 8%. Remaining cells were washed, counted, and used as stimulating cells in MLC (see Tables I-III), and as donor-specific cells for pv immunization (below).

Skin transplantation

These procedures were performed essentially as described elsewhere (3, 23). When animals received donor-specific pv immunization, 10×10^6 bone marrow derived DC were infused 36 h before skin grafting. As noted in earlier publications, technical failures (due to hemorrhage) were <10%.

Cytotoxicity and cytokine assays

In cultures used to assess induction of cytotoxicity or cytokine production, C3H responder cells were stimulated with equal numbers of mitomycin C-treated (100 μg/ml, for 45 min at 37°C) spleen stimulator cells in triplicate in αF10. Supernatants were pooled at 40 h from replicate wells and assayed in triplicate in ELISA for lymphokine production, as described in detail elsewhere (4), using cytokine capture Abs and enzyme-coupled developing Abs, as indicated above. Recombinant cytokines for standardization of assays were purchased from Genzyme (Cambridge, MA). Each assay reliably quantified cytokine levels in the range 40–4000 pg/ml.

When cytotoxicity was measured, cells were harvested at 5 days and pooled from replicate wells, counted, and cultured at various E:T ratios with ^{51}Cr-labeled 72-h spleen Con A blasts as target cells. Supernatants were sampled at 4 h for assessment of specific cytotoxicity.

Production and expression of an OX2:Fc fusion protein in a baculovirus expression system (1)

An immunoadhesin constructed to contain the extracellular domain of OX2, linked to a murine IgG2aFc region, was made as described elsewhere, and expressed using a Baculovirus Expression Vector System in *Spodoptera frugiperda* insect cells. This fusion protein inhibits type 1 cytokine production from T cells stimulated with allogeneic DC in vitro (1). One milligram of OX2:Fc was FITC labeled using conventional techniques.

Cell separation by biophysical means

Velocity sedimentation, a technique separating cells into populations of different size, was performed as described elsewhere (24), on splenic cells pretreated (24 h at 37°C) with LPS (1 μg/ml) at a concentration of 3×10^6 cells/ml. Cells were washed three times with medium before sedimentation. In some experiments, small (slow-sedimenting) cells were further fractionated, following labeling with PE anti-F4/80 mAb, using an anti-PE column. Recovery of $F4/80^+$ and $F4/80^-$ cells was 75% and 90%, respectively (see individual experiments below).

Statistical analysis

For comparison of DC FACS staining, or cytokine production in different groups assayed in vitro, initial ANOVAs were performed, followed by pairwise comparison of relevant groups using a Student *t* test (see legends to figures/tables).

Results

Binding of FITC-OX2:Fc to LPS-stimulated splenic cells, but not DC, and to $\alpha\beta\gamma\delta TCR^+$ cells

Twenty-four-hour LPS-stimulated fresh splenic macrophages and DC were obtained from a pool of three C57BL/6 mice, while 72-h Con A-activated PLN and PP cells were similarly prepared from a pool of three other C57BL/6 donors. Cells were harvested, washed, preincubated for 30 min at 4°C in PBS with 1% mouse serum, and thereafter stained with various different fluorescent mAbs, as shown in Fig. 1. As a control, cells were incubated independently also with a FITC-coupled mouse MOPC173 IgG2a myeloma (*lower panels* in figure). Data in Fig. 1 show the FACS profiles from one of four such studies using these different cell populations. In the *upper panels*, cells are shown stained with FITC anti-TCR (anti-αβTCR for PLN, anti-γδTCR for PP) or with PE anti-CD80 (splenic macrophages and DC).

It is clear that subpopulations of activated $\alpha\beta TCR^+$ cells and splenic macrophages bound the FITC-OX2:Fc, while no significant staining was seen with LPS-activated splenic DC. A total of >80% of activated PP $\gamma\delta TCR^+$ cells stained with FITC-OX2:Fc. When similar studies were performed using resting cells (no LPS or Con A activation), or in the presence of a 5-fold excess of unlabeled OX2:Fc, no binding of FITC-OX2:Fc above control was seen for any cell population (data not shown). In the work that follows, we have concentrated on characterizing the population of splenic cells binding FITC-OX2:Fc.

Splenic cells binding FITC-OX2:Fc are physically distinguishable from $OX2^+$ cells

It was intriguing to us that a DC-enriched cell population did not bind FITC-OX2:Fc (Fig. 1), while splenic macrophages did, because earlier data had suggested the existence of an immunoregulatory $OX2^+$ DC that could modulate stimulation induced by OX2- DC (7). Accordingly, we next asked whether $OX2^+$- and OX2:Fc-binding cells could be further characterized. Forty-eight-hour LPS-stimulated, T-depleted, spleen cells were subjected to fractionation by size using unit gravity sedimentation techniques (24), and different cell populations were incubated with FITC-OX2:Fc, PE (or FITC) anti-OX2, and PE anti-CD80, again after first preincubation with mouse serum (see above). Data shown in Fig. 2 indicate $FITC\text{-}OX2{:}Fc^+CD80^+$ cells, $FITC\text{-}OX2{:}Fc^-$

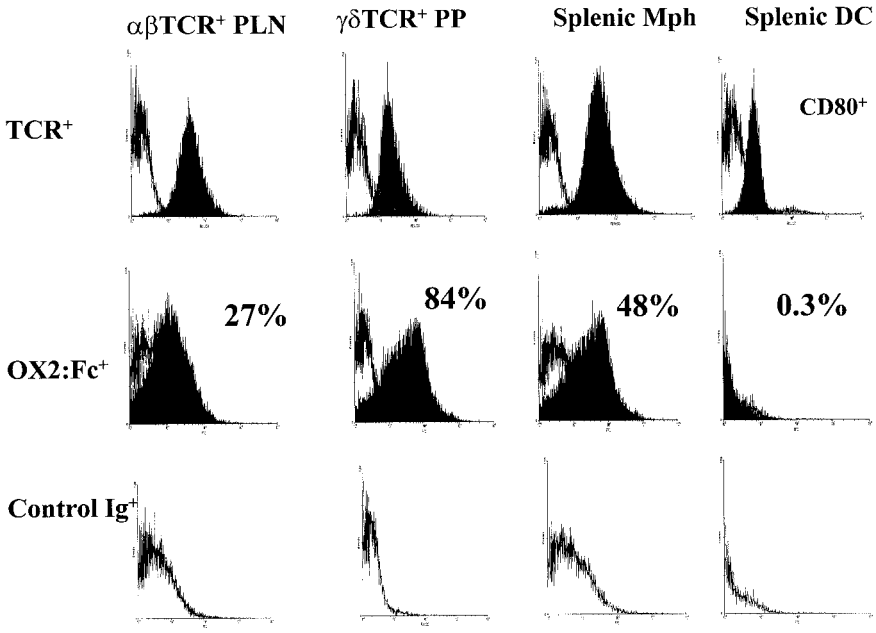

FIGURE 1. FACS analysis of 24-h LPS-stimulated fresh splenic macrophages and DC, or 72-h Con A-activated PLN and PP cells. Cells were preincubated for 30 min at 4°C in PBS with 1% mouse serum before staining with the FITC anti-TCR or PE anti-CD40 (*upper panels*); FITC-OX2:Fc (*middle panels*); or, as control, FITC-coupled mouse MOPC173 myeloma (Ig2a). *Upper panels*, Cells stained with control Igs are shown in the unshaded area. *Middle panels*, The unshaded area represents a FACS profile for cells in the absence of addition of FITC-OX2:Fc.

$CD80^+$ cells, FITC-$OX2{:}Fc^+CD80^-$, $OX2^+CD80^+$, $OX2^+CD80^-$, and $OX2^-CD80^+$ cells. No cells were detected that bound both FITC-OX2:Fc and PE anti-OX2 (data not shown).

It is clear from these data that $CD80^+$ and $CD80^-$ FITC-OX2:Fc-binding cells exist, and that these are found predominantly in a slow-sedimenting splenic population that contains some 50% of this splenic pool. In addition, both $CD80^+$ and $CD80^-$ $OX2^+$ cells exist, but in contrast to the OX2:Fc-binding cells, these are found in the faster-sedimenting cell population, making up <10% of this splenic pool.

Stimulation of OX2:Fc-binding cells with OX2:Fc delays graft rejection in vivo, and inhibits alloreactivity in vitro

Earlier studies have shown that along with an increase in graft survival, infusion of OX2:Fc into grafted mice leads to a polarization of cytokine production in lymphoid cells toward increased production of IL-4, IL-10, and TGF-β, with decreased production of IL-2, IFN-γ, and TNF-α. In vitro, incubation of allostimulated cells in the presence of OX2:Fc led to inhibition of development of CTL in vitro, decreased proliferation, and decreased production of the cytokines IL-2 and IFN-γ (1). Because membrane-bound OX2 itself lacks any significant signaling domains in the intracellular region (25), we had earlier speculated that immunoregulation occurred following engagement of OX2L on the receptor-bearing cell (1, 4, 25). To investigate whether immunoregulation induced in the presence of OX2:Fc was indeed enhanced by the presence of $OX2L^+$ cells, we performed the following study.

Slow-sedimenting spleen cells (capable of binding FITC-OX2:Fc; see Fig. 2) and fast-sedimenting ($OX2^+$) cells were obtained, as described above, from a pool of LPS-stimulated T-depleted spleen cells from six C57BL/6 mice. A control population of cells (intermediate sedimentation velocity <3% $OX2^+$; <4% binding of FITC-OX2:Fc) was harvested also. Groups of seven C3H mice received C57BL/6 skin grafts, along with cells from these different spleen pools, with or without infusion of OX2:Fc (10 μg/mouse; two doses only at 48-h intervals). Skin graft survival was followed daily (see Fig. 3).

In a separate study, C3H spleen cells were cultured with mitomycin C-treated C57BL/6 DC, again in the presence/absence of pools of the same splenic cells, with/without additional OX2:Fc protein. CTL (day 5) and cytokines (in the culture supernatant at 40 h) were measured in standard fashion. Data from one of three such studies are shown in Table I.

As described in our earlier report, infusion of OX2:Fc alone led to increased skin allograft survival. Only the $OX2^+$ large cell pool (sedimenting at >6 mm/h) produced any prolongation of survival following infusion of the pools of splenic cells tested alone (see Fig. 3*a*). As predicted from data using anti-OX2 mAbs in pv immunized mice, infusion of anti-OX2 into mice receiving these cells abolished the increase in graft survival seen (R. M. G., unpublished data). However, infusion of OX2:Fc, in association with cells capable of binding FITC-OX2:Fc ($OX2L^+$), the small cells (slow-sedimenting pool), led to the most pronounced increased survival seen. These data were recapitulated by in vitro studies. Addition of OX2:Fc alone to the culture medium inhibited production of CTL and type 1 cytokines (IL-2, IFN-γ). When used alone, the fast-sedimenting population of cells ($OX2^+$) caused some inhibition in the assays measured. However, this was only modestly augmented by further addition of soluble OX2:Fc (percentage of change from group with no OX2:Fc, 58% for CTL, $p < 0.05$; 46% for cytokines, $p > 0.05$). In contrast, cells from the slow-sedimenting spleen pool (which bind FITC-OX2:Fc) produced little inhibition of CTL/cytokine activity by themselves, but caused profound inhibition in both assays in the presence of additional OX2:Fc (see Table I: percentage of change from group with no OX2:Fc was 93% for CTL, $p < 0.01$; 88% for cytokines, $p < 0.01$).

Suppressive activity in splenic cells binding FITC-OX2:Fc resides in F4/80$^+$ cells

In an earlier study, we had found that following pv immunization, adoptive transfer of inhibition of alloresponses was possible using an $F4/80^+$ cell population (20). Similarly, in a model of immune deviation following Ag inoculation to the anterior chamber of the eye, inhibition was adoptively transferred by $F4/80^+$ cells (26). We speculated that the inhibition seen using the pool of slow-sedimenting $OX2L^+$ cells described above might be associated with this $F4/80^+$ cell population. Accordingly, T-depleted, LPS-stimulated spleen cells were fractionated as above, and following

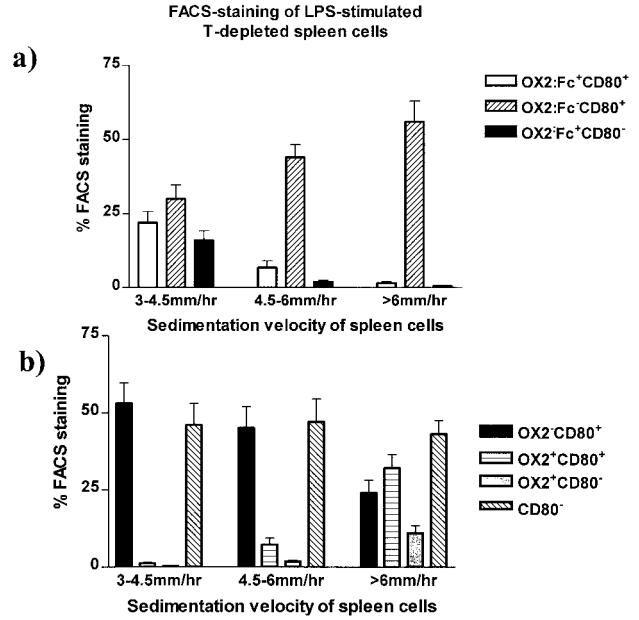

FIGURE 2. Characterization of $OX2^+$- and OX2:Fc-binding cells in 48-h LPS-stimulated, T-depleted, spleen cells after fractionation of the cells by size using unit gravity sedimentation techniques (24). The different cell populations shown were incubated with FITC-OX2:Fc, PE (or FITC) anti-OX2, and PE anti-CD80, again after first preincubation with mouse serum (see Fig. 1). *a*, Data show staining of cells with FITC-OX2:Fc. *b*, Data show staining with FITC anti-OX2. No cells were detected that bound both FITC-OX2:Fc and PE anti-OX2 (data not shown).

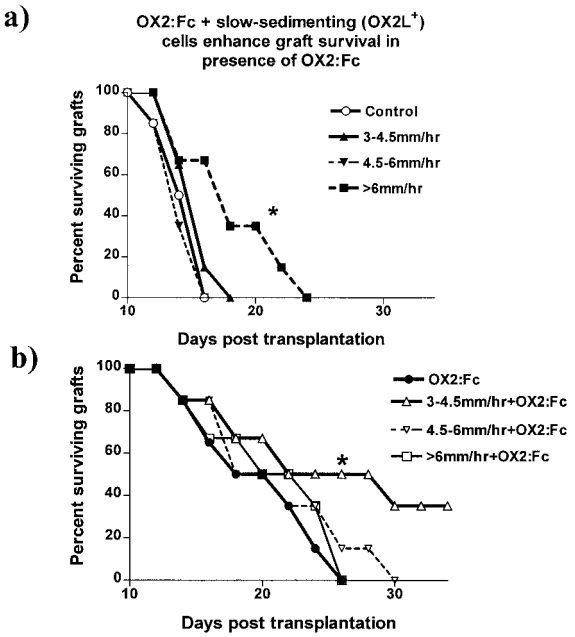

FIGURE 3. Skin allograft survival after infusing slow-sedimenting spleen cells (capable of binding FITC-OX2:Fc; see Fig. 2) along with soluble OX2:Fc. Various populations of spleen cells were obtained from a pool of LPS-stimulated T-depleted spleen cells from six C57BL/6 mice (see Fig. 2). Groups of seven C3H mice received C57BL/6 skin grafts, along with cells from these different spleen pools, with or without infusion of OX2:Fc (10 μg/mouse; two doses only at 48-h intervals). Skin graft survival was followed daily. *a*, Data show survival for groups receiving LPS-spleen cell subpopulations alone; $*$, $p < 0.05$ (Mann-Whitney *U* test), compared with control (no additional cells or OX2:Fc). *b*, Data show results for groups receiving cells + OX2:Fc; $*$, $p < 0.05$, compared with other groups.

separation into small ($OX2L^+$: 3–4.5 mm/h sedimentation velocity) or large ($OX2^+$: >6 mm/h sedimentation velocity) cells, we labeled the cell population with PE anti-F4/80 Ab (~10% cells were stained) and used anti-PE magnetic bead separation to recover $F4/80^+$ and $F4/80^-$ cells. Medium-size cells (with fewer $OX2L^+$ cells; see Fig. 2) showed ~18% staining with F4/80; large cells showed negligible staining (<4%) with F4/80 Ab (data not shown). All cell populations were then tested as in Table I for their ability to inhibit the response of C3H responder cells stimulated with C57BL/6 stimulator cells, in the presence or absence of additional OX2:Fc. Data for one of two such studies are shown in Table II.

As already shown in Table I, inhibition from large ($OX2^+$) cells is independent of exogenous addition of OX2:Fc. In contrast, no inhibition was seen using small cells until OX2:Fc was added, when this population (containing $OX2L^+$ cells) was the optimally inhibitory pool. More important, the $F4/80^+$ in this $OX2L^+$ small cell population was the most active for suppression in the context of exogenous OX2:Fc, with little such activity present in the small cell, $F4/80^-$ population.

Suppression by $OX2L^+$ cells follows direct allorecognition of MHC-incompatible APC

In vitro, slow-sedimenting cells from LPS-stimulated C57BL/6 T-depleted spleen cultures ($OX2L^+$) can be used, following engagement of OX2:Fc, to inhibit the response of C3H responder cells stimulated to recognize C57BL/6 alloantigen. To assess whether the inhibition reflects a suppressive function following engagement by OX2:Fc by $OX2L^+$ cells that leads these cells to inhibit bystander immune responses (e.g., to third-party Ags), we performed the following study. Inhibition of B10.BR anti-B10, B10 anti-B10.BR, or third-party B10.BR anti-B10.D2 alloresponses was assessed using culture systems analogous to those shown in Table I, in which the inhibitory population (of $OX2L^+$ cells) used was the slow-sedimenting pool obtained from T-depleted, LPS-stimulated, B10.Sgn spleen cells. Data for one of two such studies are shown in Table III.

It is clear from these data that while exogenous OX2:Fc inhibits CTL and cytokine production in all the allorecognition combinations used, addition of slow-sedimenting B10 ($OX2L^+$) cells increased this inhibition only when responder T cells simultaneously recognized the same (B10) cells. Bystander inhibition of a third-party (B10.BR anti-B10.D2) response by $OX2L^+$ cells did not occur, nor indeed was inhibition seen when $OX2L^+$ cells were MHC compatible with the responder cell pool (B10 anti-B10.BR combination). Thus, it seems the inhibitory signal delivered by $OX2L^+$ cells follows from their direct recognition, by responder T cells, in this case by MHC-directed TCRs on responder cells.

Discussion

Unresponsiveness to donor renal allografts following Ag-specific pv pre- or peritransplant immunization is associated with the preferential activation of type 2 rather than type 1 cytokine-producing cells (23, 27). T cell activation depends not just upon the delivery of signals via the TCR (generally in the form of Ag-MHC), but also on the appropriate delivery of costimulatory signals from APCs (28), along with the cytokine milieu in which stimulation occurs. We (4, 6, 7) and others (29) have also reported evidence that implicated another molecule, OX2, in the regulation of alloresponsiveness, and more particularly in the regulation of graft rejection following pv immunization (4). More recently, we confirmed, using species-specific mAbs to human, rat, or mouse OX2,

Table I. *A slow-sedimenting (3–4.5 mm/h) LPS-stimulated spleen cell population is stimulated in the presence of OX2:Fc to inhibit CTL and type 1 cytokine production in vitro*

LPS-Stimulated Cells Added[a]	OX2:Fc Added	Percent Lysis ^{51}Cr Targets[b]	LU_{20} (% inhibition)[c]	Cytokines in Culture (% inhibition)[d] IL-2	IFN-γ
None	–	44 ± 6.7	821	1180 ± 210	940 ± 100
None	+	13 ± 3.6*	139 (83)	360 ± 75*(69)	245 ± 40*(74)
3–4.5 mm/h	–	36 ± 6.1	446	1135 ± 190	870 ± 120
3–4.5 mm/h	+	3.2 ± 2.0**	33 (96)	140 ± 50**(88)	100 ± 40**(89)
4.5–6 mm/h	–	42 ± 5.6	545	1215 ± 220	940 ± 110
4.5-6 mm/h	+	16 ± 3.9*	174 (68)	335 ± 110*(72)	270 ± 60*(71)
>6 mm/h	–	19 ± 4.2*	211	505 ± 130*	400 ± 90*
>6 mm/h	+	8.4 ± 2.9*	82 (58)	275 ± 70*(46)	210 ± 60*(47)

[a] A total of 5×10^6 responder C3H spleen cells pooled from three 8-wk donors were cultured in triplicate with 2.5×10^6 mitomycin-c-treated C57BL/6 DC in the presence or absence of 1×10^6 cells from one of three pools of velocity-sedimented, LPS-stimulated, T-depleted spleen cells (see Fig. 3 for more details). Cultures received additional OX2:Fc protein as shown.

[b] Percent lysis (50:1, E:T) at 5 days using 1×10^4 ^{51}Cr C57BL/6 spleen ConA targets.

[c] LU_{20} are defined as the $LU/10^6$ cells, where 1 LU is defined as containing sufficient CTL to lyse 20% of targets in 4 h. Data in parentheses show percent inhibition of corresponding control cultures with no added OX2:Fc.

[d] Cytokine (pg/ml) in culture supernatants harvested at 40 h. Data in parentheses represent percent inhibition of response in control cultures (first row).

*, $p < 0.05$, compared with control cultures in first row (ANOVA followed by pair-wise *t* test).

**, $p < 0.05$, compared with all other groups (pair-wise *t* test).

that mAbs to OX2 detected on the surface of host DC may play a role in regulating cytokine production after allostimulation in vitro (19); this activity was seen using $F(ab')_2$ anti-OX2 reagents (R. M. G., in preparation).

Functional blockade of OX2 expression led to increased IL-2 production (a type 1 cytokine) after allostimulation (19). Moreover, functional blockade of OX2 expression from the time of transplantation abolished prolongation of graft survival following pv immunization (4, 6). In other experiments using an immunoadhesin prepared by genetic fusion of OX2 to a murine IgG2a Fc region (1), we reported data suggesting that a major regulatory signal from OX2 was delivered during the initiation phase of the immune response. Because OX2 itself lacks any signaling motifs in its intracellular domain(s), and docking sites for adapter signaling molecules (25), we had hypothesized that a ligand-bearing cell (hereafter referred to as $OX2L^+$) would be most relevant for immunoregulation following increased OX2 expression (1, 4). The current studies have examined this possibility, and provide data for the existence of several $OX2L^+$ cell populations, one of which has potent immunosuppressive properties after admixture with OX2:Fc itself. Note that the OX2:Fc molecule used in our studies was derived from a construct using a mutant IgG2a molecule, lacking both the complement-binding domains and the Fc-binding domains. Thus, we consider it unlikely that binding by OX2:Fc is to Fc receptors on the cell surface. In addition, cells were preincubated with an excess of murine Ig to saturate such sites (see text). Finally, LPS-stimulated splenic DC, which also possess FC receptors, do not bind OX2:Fc (see Fig. 1).

It is important to note that there is a previous report that an OX2 ligand-bearing population was present in a macrophage cell preparation (18). In these studies, in a fashion somewhat similar to that described above, a soluble chimeric protein with the extracellular domains of OX2 engineered onto domains (3 + 4) of rat CD4 Ag was used to screen for an OX2-binding cell population. There was no evidence for the presence of OX2-binding cells in the T cell populations studied in this study, which is in contradistinction to the data above (see Fig. 1). It must be acknowledged, however, that there are no data existing concerning the identity of the ligand for OX2 on the surface of these different cell populations, which in itself may contribute to unique functional properties in these different cells. Moreover, the very existence of a receptor, with an immunoregulatory role, for OX2 on such a high percentage of activated $\gamma\delta TCR^+$ cells is consistent with much earlier data from

Table II. *$F4/80^+$, slow-sedimenting (3–4.5 mm/h), LPS-stimulated spleen cells stimulated in the presence of OX2:Fc provide optimal inhibition of CTL and type 1 cytokine production in vitro*[a]

LPS-Stimulated Cells Added	OX2:Fc Added	Percent Lysis ^{51}Cr Targets (50:1, E:T)	Cytokines in Culture (pg/ml) IL-2	IFN-γ
None	–	31 ± 4.2	910 ± 160	1060 ± 150
None	+	17 ± 3.2*	460 ± 70*	510 ± 90
3–4.5 mm/h:Unfx	–	30 ± 4.1	915 ± 150	1040 ± 140
3–4.5 mm/h:Unfx	+	6.9 ± 2.3**	240 ± 55**	230 ± 45**
3–4.5 mm/h:$F4/80^+$	–	28 ± 5.3	935 ± 160	1050 ± 160
3–4.5 mm/hr $F4/80^+$	+	3.0 ± 1.7**	90 ± 30**	105 ± 35**
3–4.5 mm/h:$F4/80^-$	–	27 ± 4.8	915 ± 150	990 ± 145
3–4.5 mm/h:$F4/80^-$	+	16 ± 3.2*	380 ± 50*	390 ± 40*
>6 mm/h	–	14 ± 3.7*	310 ± 80*	390 ± 85*
>6 mm/h	+	8.6 ± 2.9*	270 ± 75*	230 ± 55*

[a] As for Table I, 5×10^6 responder C3H spleen cells pooled from three 8-wk donors were cultured in triplicate with 2.5×10^6 mitomycin-c-treated C57BL/6 DC in the presence or absence of 1.5×10^6 (or 3×10^5 for the $F4/80^+$ pool) cells from velocity-sedimented, LPS-stimulated, T-depleted spleen cells. Cultures received additional OX2:Fc protein as shown. Further separation of small cells into $F4/80^+$ and $F4/80^-$ populations used an anti-PE column and PE-anti-F4/80 (see *Materials and Methods*). Total recovery was 80%, and recovery of the $F4/80^+$ population (10% of the 3–4.5 mm/h pool) was 70%.

*, $p < 0.05$, compared with control cultures in first row (ANOVA followed by pair-wise *t* test)

**, $p < 0.05$, compared with all other * groups (pair-wise *t* test).

Table III. *Inhibition of CTL induction by slow-sedimenting (3–4.5 mm/h) LPS-stimulated spleen cells in the presence of OX2:Fc depends upon their being MHC-matched with allo-stimulatory DC*

LPS-Stimulated[a] Cells Added	OX2:Fc Added	Percent Lysis ^{51}Cr Targets (50:1 E:T ratio)[b]		
		B10.BR anti-B10.Sgn ($H2^{b/b}$)	B10.Sgn anti-B10.BR ($H2^{k/k}$)	B10.BR anti-B10.D2 ($H2^{d/d}$)
None	–	40 ± 5.3	43 ± 5.5	52 ± 6.3
None	+	12 ± 3.2*	13 ± 3.3*	17 ± 4.4*
3–4.5 mm/h	–	37 ± 6.4	42 ± 6.0	49 ± 5.9
3–4.5 mm/h	+	4.6 ± 2.2**	12 ± 3.4*	16 ± 4.2*
4.5–6 mm/h	–	39 ± 6.3	40 ± 4.9	44 ± 5.6
4.5–6 mm/h	+	13 ± 3.4*	13 ± 2.9*	15 ± 4.0*
>6 mm/h	–	17 ± 4.3*	32 ± 4.4*	28 ± 4.9*
>6 mm/h	+	7.2 ± 2.4*	10 ± 2.3*	13 ± 3.8*

[a] A total of 5×10^6 responder spleen cells were pooled from three 8-wk B10.BR (anti-B10, anti-B10.D2 response) or B10.Sgn (anti-B10.BR response) donors. Cells were cultured in triplicate with 2.5×10^6 mitomycin-c-treated allogeneic DC in the presence or absence of 1×10^6 cells from one of three pools of velocity-sedimented, LPS-stimulated, T-depleted, B10.Sgn spleen cells (Table I). Some cultures received additional OX2:Fc protein as described in *Materials and Methods*.

[b] Percent lysis (50:1, E:T) at 5 days using 1×10^4 ^{51}Cr spleen ConA targets.

*, $p < 0.05$, compared with control cultures in first row (ANOVA followed by pair-wise *t* test).

**, $p < 0.05$, compared with all other groups (pair-wise *t* test).

this laboratory concerning the potential role for such cells in regulation of transplant rejection following pv immunization (20).

In studies of the adoptive transfer of increased graft survival in mice pretreated with donor-specific alloantigen infusion via the pv (a process that leads to up-regulation of OX2 expression on DC (4)), we reported that an F4/80$^+$ adherent spleen cell population was associated with the suppression of graft rejection. Similarly, Streilein et al. (26) have reported that F4/80$^+$ cells are implicated in adoptive transfer of tolerance following Ag infusion into the anterior chamber of the eye. F4/80$^+$ cells were found in the slow-sedimenting pool of cells found after LPS stimulation of T-depleted spleen cells. By further purification of these OX2L$^+$ cells into F4/80$^+$ and F4/80$^-$ cells, we have been able to show that the inhibition of alloresponses seen using OX2L$^+$ cells mixed with OX2:Fc can be accounted for by the F4/80$^+$ cell pool (see Table II).

One could envisage a model in which following OX2 engagement of OX2L on the surface of F4/80$^+$ cells, the latter become nonspecifically suppressive (e.g., by release of cytokines) to T cells activated in their vicinity. However, the data shown in Table III argue against such a model. In this study, we show that using a B10-suppressive cell pool (OX2L$^+$), the suppression of alloimmunity seen is directed to T cells recognizing the alloantigen expressed on those OX2L$^+$ cells. Even responses using responder cells, MHC compatible with the OX2L$^+$, F4/80$^+$ inhibitory cells are unaffected, as are third-party immune responses. We conclude that T responder cells are down-regulated following direct cell:cell MHC-restricted recognition of the inhibitory OX2L$^+$ cells. In the case of immunity to nominal Ag (rather than alloantigen), we suggest that the OX2L$^+$ cell must itself present the Ag to the responder lymphocyte in order for the latter to receive its tolerizing signal. This mechanism presumably represents a necessary safeguard to ensure that this form of immunotolerance does not cause bystander nonspecific immunosuppression in the individual.

There has been considerable interest in the heterogeneity of DC and the significance of this to immunization/tolerance induction. DC are heterogeneous in terms of origin, cell surface phenotype, turnover in vivo, and possibly function (30, 31). It is known that the induction of immunity (vs tolerance) following Ag presentation is 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)) (32–34). Thus, CD80, CD86, and CD40 are all implicated as costimulator molecules in transplantation (12–17). From studies focusing on a role for the molecule OX2 in induction of unresponsiveness, we concluded that discrete populations of DC preferentially expressing OX2 were uniquely capable of inducing tolerance to mouse allograft tissue/organs (7). The data shown above add further complexity to this model. We now suggest that a distinct population of cells, expressing OX2L, are themselves ultimately engaged in the delivery of key immunoregulatory signals following engagement (by OX2) on the cell surface. Interestingly, in a recent study, Vremec et al. (35) have described a novel CD4$^+$ DC that also expresses F4/80, as a component of a so-called lymphocyte-derived DC pool, a population previously thought to be implicated in tolerance induction. No function was described for this population in this study, and it remains to be assessed whether this F4/80$^+$ population is the OX2L$^+$ cell we have described in this study. The potential role manipulation of such OX2$^+$/OX2L$^+$ cells has for transplantation, autoimmunity, and other diseases of perturbed immunoregulation remains to be determined.

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